

Potassium channel mutants of the yeast *Saccharomyces cerevisiae* and their use for screening eukaryotic potassium channels

The invention relates to processes for identifying inhibitors and activators of eukaryotic potassium channels, in which a mutated *S. cerevisiae* cell is used whose endogenous potassium channels TRK1, TRK2 and TOK1 are not expressed functionally, but which heterologously expresses a eukaryotic potassium channel to be studied. Other subject matters of the invention are mutated *S. cerevisiae* cells which do not express TRK1, TRK2 and TOK1, and the preparation and use of these mutated *S. cerevisiae* cells.

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Each cell is enclosed by a plasma membrane with a thickness of approximately 6 - 8 nm. This membrane determines the cell's dimensions and separates the cell content from its environment. All biomembranes are composed of a connected bilayer of lipid molecules, which bilayer accommodates a variety of membrane proteins. While the lipid bilayer determines the basic structure of biomembranes, the proteins are responsible for most of their functions. Owing to its hydrophobic interior, the lipid bilayer acts as an impermeable barrier for most polar molecules. Only membrane proteins such as receptors, ion channels and transporters allow controlled ion flux and the transport of polar molecules (Alberts *et al.*, 1995). Thus, proteins contribute to different ion concentrations in the cell's interior and its environment and govern the entry of nutrients and the exit of breakdown products. Most of the membrane proteins span the plasma membrane repeatedly, as do the ion channels, which thus belong to the group of the integral membrane proteins. These proteins have both hydrophobic regions, which span the lipid bilayer, and hydrophilic sections, which are exposed to the aqueous medium on either side of the membrane. Ion channels are found in all cells and, in nerve cells, are responsible for the generation of action potentials (Alberts *et al.*, 1995). Ion channels can be differentiated on the basis of their different ion selectivity and with reference to their different opening and closing mechanisms.

30 Potassium channels are ubiquitous membrane proteins found both in excitable and in nonexcitable cells (for review see (Jan, L. Y. et al., 1997). Open potassium channels shift the membrane potential closer to the potassium equilibrium potential and thus away from the threshold potential for triggering an action potential. Thus, potassium

channels strengthen the resting membrane potential, repolarizing the cell and in this way determine the length of the frequency of action potentials (Sanguinetti, M. C. et al., 1997; Wilde, A. A. et al., 1997; Wang, Q. et al., 1998). Owing to these functions, potassium channels also constitute the molecular cause for the generation of a number of pathological situations and are thus an interesting target for the development of therapeutical agents.

The yeast *Saccharomyces cerevisiae* (hereinbelow *S. cerevisiae*) has three potassium channels, namely TRK1, TRK2 and TOK1. The potassium channel TRK1 (YJL129c) belongs to the family of the "major facilitator" potassium permeases and, being a high-affinity potassium transporter, is responsible for the influx of potassium ions from the medium into the cell (Gaber, R. F. et al., 1988; Ko, C. H. et al., 1990; Ko, C. H. et al., 1991). The deletion mutant $\Delta trk1$ is viable and highly polarized on at least 10 mM K^+ (Gaber, R. F. et al., 1988; Madrid, R. et al., 1998). A $\Delta trk1$ strain does not survive on 1 mM K^+ (Gaber, R. F. et al., 1988).

The potassium channel TRK2 (YKR050w) also belongs to the family of the "major facilitator" potassium permeases and, being a low-affinity potassium transporter, is responsible for the influx of potassium ions from the medium into the cell (Ko, C. H. et al., 1990; Ko, C. H. et al., 1991; Madrid, R. et al., 1998). The phenotype of the $\Delta trk2$ deletion mutant is less pronounced than in the case of the $\Delta trk1$ mutant. A $\Delta trk2$ strain also survives on 1 mM K^+ (Ko, C. H. et al., 1990; Madrid, R. et al., 1998).

The potassium channel TOK1 (also known as DVK1 or YORK) is responsible for the influx of potassium ions from the medium into the cell (Ketchum, K. A. et al., 1995; Fairman, C. et al., 1999). However, the direction of the ion fluxes is reversible, and, depending on the culture conditions, can therefore also take the opposite direction (Fairman, C. et al., 1999).

The deletion mutant $\Delta trk1 \Delta trk2$ has already been described repeatedly (Ko, C. H. et al., 1990; Ko, C. H. et al., 1991; Madrid, R. et al., 1998; Fairman, C. et al., 1999).

In the past, this mutant was also used for identifying and describing K^+ channels of higher eukaryotes by complementation of the phenotype. Described to date is the

complementation by the *inward rectifier* channels KAT1 cDNA (*Arabidopsis thaliana*), HKT1 cDNA (*Triticum aestivum*), IRK1 (*Mus musculus*) and HKT1 K⁺/Na⁺ transporters (*Triticum aestivum*) (Tang, W. et al., 1995; Smith, F. W. et al., 1995; Goldstein, S. A. et al., 1996; Nakamura, R. L. et al., 1997). In addition, it has been described that the overexpression of TOK1 and its homologue ORK1 from *Drosophila melanogaster* in yeast cells can complement the growth deficiency of the $\Delta trk1 \Delta trk2$ mutant (Fairman, C. et al., 1999).

However, the study of a large number of eukaryotic potassium channels and the identification of substances which can modify the activity of the potassium channels is difficult since, for example, the human channels HERG1 or Kv1.5 cannot complement the lethal phenotype of $\Delta trk1 \Delta trk2$ on 5 mM KCl. Thus, no screening is possible.

The invention relates to a process for identifying inhibitors of a eukaryotic potassium channel, in which

- a) a mutated *S. cerevisiae* cell is used which does not express the three endogenous potassium channels TRK1, TRK2 and TOK1;
 - b) a eukaryotic potassium channel is expressed heterologously in this mutated *S. cerevisiae* cell;
 - c) the mutated *S. cerevisiae* cell is incubated together with a substance to be tested;
- and
- d) the effect of the substance to be tested on the eukaryotic potassium channel is determined.

In the mutated *S. cerevisiae* cell used in the method, the genes TRK1, TRK2 and TOK1 (SEQ ID NO. 1, SEQ ID NO. 2 and SEQ ID NO. 3)

are switched off ($\Delta trk1$, $\Delta trk2$, $\Delta tok1$), preferably by knock-out, it being preferred for large portions of the genes to be deleted.

The eukaryotic potassium channel used in the process is the potassium channel to be studied, the channel for which inhibitors or activators are to be identified.

For example, the eukaryotic potassium channel is a human HERG1, a human Kv1.5, a human ROMK2 or gplRK1 (guinea pig) channel. The eukaryotic potassium channel preferably has the natural sequence of the potassium channel in question, for example 5 encoded by one of the sequences SEQ ID NO. 4, SEQ ID NO. 5, SEQ ID NO. 7 (ROMK2) or SEQ ID NO. 6. However, the natural sequence of the potassium channel can also be modified, for example mutated.

Preferably, the nucleotide sequence encoding the eukaryotic potassium channel is integrated into a yeast expression plasmid, for example p423 GPD3 or a vector, for 10 example of the pRS 42x or pRS 32x series, and the recombinant expression plasmid is introduced into the mutated *S. cerevisiae* cell.

The process is intended to identify substances which have an effect on the eukaryotic potassium channel. These substances inhibit the growth of the mutated *S. cerevisiae* 15 cell. A substance to be studied which inhibits the heterologously expressed eukaryotic potassium channel causes the mutated *S. cerevisiae* cell – since it does not express endogenous potassium channels - to divide and multiply with greater difficulty or more slowly or, in a particular embodiment of the invention, to die.

20 The effect of the substance to be tested can be determined for example directly by measuring the optical density at 600 nm or with the aid of a growth reporter which is expressed constitutively in the mutated *S. cerevisiae* cell. The constitutively expressed growth reporter preferably encodes a protein which either shows fluorescence or luminescence itself or which participates in a reaction which gives a fluorescence or 25 luminescence signal. The sequence encoding the growth reporter is preferably of a vector. Suitable growth reporters are, for example, the LacZ gene for β -galactosidase or acid phosphatase PH03, both of which are expressed under the control of a constitutive yeast promoter. The measurable fluorescence or luminescence allows conclusions regarding the cell count of the mutated *S. cerevisiae* cells. If no, or less, 30 fluorescence or luminescence is measured, then the sample in question contains fewer mutated *S. cerevisiae* cells. If fewer mutated *S. cerevisiae* cells are present, then the substance to be tested has an inhibitor effect on the eukaryotic potassium channel.

The processes described can be automated with particular ease and carried out in parallel for a multiplicity of substances to be tested. In particular embodiments of the invention, two or more processes are carried out in a comparative fashion, where two or more mutated *S. cerevisiae* cells are analyzed in a comparative fashion. These mutated *S. cerevisiae* cells are preferably incubated together with the same amount of substance to be tested, but express the eukaryotic potassium channel in question to a different extent. In another particular embodiment of the invention, mutated *S. cerevisiae* cells which express the eukaryotic potassium channel in question to the same extent, but which are incubated together with different amounts of substance to be tested, are analyzed in a comparative fashion.

Subject matter of the invention is also a mutated *S. cerevisiae* cell in which the endogenous potassium channels TRK1, TRK2 and TOK1 are not expressed. A further embodiment relates to a mutated *S. cerevisiae* cell in which the genes TRK1, TRK2 and TOK1 are switched off; these genes have preferably been removed by knock-out in their entirety or in part, or have been mutated. A further embodiment relates to a mutated *S. cerevisiae* cell which is deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Mascheroder Weg 1b, D-38124 Braunschweig) in compliance with the provisions of the Budapest Treaty on the International recognition of the deposit of microorganisms for the purposes of patent procedure; deposit number DSM 13197.

A particular embodiment of the invention relates to a mutated *S. cerevisiae* cell which heterologously expresses a eukaryotic potassium channel, the eukaryotic potassium channel preferably being a human potassium channel, for example a HERG1, Kv1.5 or gpiRK1 or a human Kv 4.3 [Genbank Accession Number AF 187963], TASK (Genbank Accession Number AF 006823) or ROMK2 [Genbank Accession Number U 12542] and where the potassium channel has the natural sequence or can be mutated.

The invention also relates to a process for the preparation of a mutated *S. cerevisiae* cell which does not express the potassium channels TRK, TRK2 and TOK1, the genes TRK1, TRK2 and TOK1 having been destroyed or deleted by knock-out.

The mutated *S. cerevisiae* cell can be used for example in processes for identifying substances which inhibit or activate the activity of the eukaryotic potassium channel, or it can be part of a test kit which can be used for example for determining toxic substances.

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The invention also relates to a process for identifying activators of a eukaryotic potassium channel, in which

- a) a mutated *S. cerevisiae* cell is used which does not express the three endogenous potassium channels TRK1, TRK2 and TOK1;
- 10 b) a eukaryotic potassium channel is expressed heterologously in this mutated *S. cerevisiae* cell;
- c) the mutated *S. cerevisiae* cell is incubated together with a substance to be tested;
- and
- 15 d) the effect of the substance to be tested on the eukaryotic potassium channel is determined.

The invention furthermore relates to a process for identifying activators of a eukaryotic potassium channel, in which

- 20 a) a mutated *S. cerevisiae* cell is used which does not express the three endogenous potassium channels TRK1, TRK2 and TOK1;
- b) a eukaryotic potassium channel is expressed heterologously in this mutated *S. cerevisiae* cell;
- c) the mutated *S. cerevisiae* cell is incubated together with a substance to
- 25 be tested in the presence of an inhibitor of the eukaryotic potassium channel;
- and
- d) the effect of the substance to be tested on the eukaryotic potassium channel is determined.

30

The invention also relates to a process for the preparation of a medicament, in which

- a) an Inhibitor of a eukaryotic potassium channel is identified,

- b) the Inhibitor is prepared or isolated by known chemical processes, and
- c) physiologically acceptable additives are added to the inhibitor.

The invention also relates to a process for the preparation of a medicament, in which

- 5 a) an activator of a eukaryotic potassium channel is identified,
- b) the activator is prepared or isolated by known chemical processes, and
- c) physiologically acceptable additives are added to the activator.

Figures:

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Figure 1: Diagnostic PCR for verifying the triple knock-out. Explanation of the rows/lanes in the gel, see text, Example 2, triple knock-out.

Figure 2: Growth of strains YM168 ($\Delta trk1 \Delta trk2$) and YM182 ($\Delta trk1 \Delta trk2 \Delta tok1$) on
15 DPM medium with defined KCl concentrations at pH 6.5.

Figure 3: Growth of strains YM189 and YM190 (in $\Delta trk1 \Delta trk2$), and YM194 and
YM195 (in $\Delta trk1 \Delta trk2 \Delta tok1$) on DPM medium with 5 mM KCl + 2 mM RbCl at pH 6.5.

20 Figure 4: Growth of strains YM189 and YM191 (in $\Delta trk1 \Delta trk2$), and YM194 and
YM196 (in $\Delta trk1 \Delta trk2 \Delta tok1$) on DPM medium with 5 mM KCl + 2 mM CsCl at pH 6.5.

Figure 5: Growth of strains YM194 and YM195 (in $\Delta trk1 \Delta trk2 \Delta tok1$) in DPM medium
with 5 mM KCl + 1 mM RbCl at pH 6.5. („KON“ = control)

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Figure 6: Growth of strains YM194 and YM196 (in $\Delta trk1 \Delta trk2 \Delta tok1$) in DPM medium
with 5 mM KCl + 1 mM CsCl at pH 6.5. („KON“ = control)

Figure 7: Expression of the human potassium channel HERG1 in the triple mutant
30 $\Delta trk1 \Delta trk2 \Delta tok1$ in DPM -HIS/-TRP 5mM KCl medium in 96-well ELISA plates in the
presence of 0.5 mM CsCl as activator.

The various inhibitors were employed at a final concentration of in each case 30µM. To measure the cell density, a commercially available LacZ reporter system pYX232 by Ingenius (cat. No. MBV-032-10) was transformed into the yeast strains to be studied. Expression of the LacZ reporter gene was under the control of the constitutive

5 *Saccharomyces cerevisiae* promotor TPI for the triose phosphate isomerase gene.

The LacZ enzyme activity was measured via detecting the luminescence after 24 hours' growth (density of the starter culture: 0.01 OD₆₂₀) using a commercially available assay system by TROPIX. The values correspond to the average of in each case 4 measurements ±SD. The two different assays were carried out independently

10 of each other on two different days.

Figure 8: Expression of the human potassium channel HERG1 in the triple mutant $\Delta trk1 \Delta trk2 \Delta tok1$ in DPM -HIS 5mM KCl medium in 96-well ELISA plates in the presence of 0.5 mM CsCl as activator.

15 The various inhibitors were employed at a final concentration of in each case 30µM. The cell density was measured after 38 hours' growth (density of the starter culture: 0.03 OD₆₂₀) via determination of the optical density at a wavelength of 620 nm. The values corresponded to the average of in each case 4 measurements ±SD.

20 Figure 9: Growth of the *Saccharomyces cerevisiae* wild-type strain in DPM -HIS/-TRP 5mM KCl medium in 96-well ELISA plates in the presence of 0.5 mM CsCl. The various inhibitors were employed at a final concentration of in each case 30µM. To measure the cell density, a commercially available LacZ reporter system pYX232 by Ingenius (cat. No. MBV-032-10) was transformed into the yeast strains to be studied.

25 Expression of the LacZ reporter gene was under the control of the constitutive *Saccharomyces cerevisiae* promotor TPI for the triose phosphate isomerase gene. The LacZ enzyme activity was measured via detecting the luminescence after 24 hours' growth (density of the starter culture: 0.01 OD₆₂₀) using a commercially available assay system by TROPIX.

30 The values correspond to the average of in each case 4 measurements ±SD. The two different assays were carried out independently of each other on two different days.

Figure 10: Growth of the *Saccharomyces cerevisiae* wild-type strain in DPM medium in 96-well ELISA plates in the presence of 5mM KCl or in the presence of 80mM KCl.

The inhibitors Ziprasidone and Pimozide were employed at a final concentration of in each case 30µM. The cell density was measured after 24 hours' growth (density of the starter culture: 0.01 OD₆₂₀) via determination of the optical density at a wavelength of 620 nm. The values corresponded to the average of in each case 4 measurements ±SD.

10 Figure 11: Expression of the human potassium channel HERG1 in triple mutant $\Delta trk1\Delta trk2\Delta tok1$, and in the double mutant $\Delta trk1\Delta trk2$ on DPM -HIS medium in the presence of 5mM KCl and 0.5 mM CsCl as activator.

1: Growth of the triple mutant $\Delta trk1\Delta trk2\Delta tok1$ upon expression of the blank vector p423GPD as negative control. 2: Growth of the triple mutant $\Delta trk1\Delta trk2\Delta tok1$ upon expression of p423GPD-TRK1 as positive control. 3: Growth of the triple mutant $\Delta trk1\Delta trk2\Delta tok1$ upon expression of p423GPD-HERG1. 4: Growth of the double mutant $\Delta trk1\Delta trk2$ upon expression of p423GPD-HERG1. The vectors and constructs used are explained in the patent application (see pages 12 et seq. and 15 et seq.).

20 Figure 12: Expression of the human potassium channel Kv1.5 in triple mutant $\Delta trk1\Delta trk2\Delta tok1$, and in the double mutant $\Delta trk1\Delta trk2$ on DPM -HIS medium in the presence of 5mM KCl and 2 mM RbCl as activator.

1: Growth of the triple mutant $\Delta trk1\Delta trk2\Delta tok1$ upon expression of the blank vector p423GPD as negative control. 2: Growth of the triple mutant $\Delta trk1\Delta trk2\Delta tok1$ upon expression of p423GPD-TRK1 as positive control. 3: Growth of the triple mutant $\Delta trk1\Delta trk2\Delta tok1$ upon expression of p423GPD-Kv1.5. 4: Expression of the double mutant $\Delta trk1\Delta trk2$ upon expression of p423GPD-Kv1.5. The vectors and constructs used are explained in the patent application (see pages 12 et seq. and 15 et seq.).

Figure 13: Expression of the human potassium channel ROMK2 and of the yeast vector p423GPD as negative control in the triple mutant $\Delta trk1\Delta trk2\Delta tok1$ in DPM -HIS 5mM KCl medium in 96-well ELISA plates.

The cell density was measured after 24 hours' growth (density of the starter culture: 5 0.01 OD₆₂₀) via determination of the optical density at a wavelength of 620 nm. The values corresponded to the average of in each case 4 measurements \pm SD.

Figure 14: Plasmid map of p423 GPD-ROMK2.

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Examples:

Materials and strains

15 Media

YPD (complete yeast medium): 1% Bacto yeast extract, 2% Bacto peptone, 2% Bacto agar, 2% glucose.

SC (synthetic complete) Medium: 0.67% Bacto yeast nitrogen base, amino acids, 2% glucose.

20 Sporulation medium: 1% potassium acetate, amino acids.

5-FOA medium: 0.67% Bacto yeast nitrogen base, amino acids, Uracil (50 μ g/ml), 2% sugar (galactose or glucose), 0.1% 5-FOA

All media are described in: (Fink, G. R. et al., 1991)

25 Amino acid dropout mix:

L-alanine 2 g; L-arginine 2 g; L-asparagine*H₂O 2.27 g; L-aspartic acid 2 g;

L-cysteine*HCl 2.6 g; L-glutamine 2 g; L-glutamic acid 2 g; glycine 2 g; myoinositol 2 g;

L-isoleucine 2 g; L-methionine 2 g; PABA 0.2 g; L-phenylalanine 2 g; L-proline 2 g;

L-serine 2 g; L-threonine 2 g; L-tyrosine 2 g; L-valine 2 g.

30

Stock solutions for marker amino acids:

	mM	g/l	
Adenine (100x)	30	5.53	heating (up to not more than 60°C)
Leucine (60x)	100	13.12	heating
Lysine (100x)	100	18.26	-
Histidine (200x)	60	12.57	-
Tryptophan (100x)	40	8.17	-
Uracil (100x)	20	2.24	heating in 0.5% NaHCO ₃ solution

Vitamin stock (50 ml): biotin 20 µg/l; calcium pantothenate 40 µg/l; thiamine 40 µg/l.

5 Defined potassium medium (DPM): for 1.5 l (2x stock):

(NH ₄) ₂ HPO ₄	8 mM	3.2 g
(NH ₄) ₂ SO ₄	29 mM	11.5 g
MgSO ₄	2 mM	0.8 g (or 6 ml of 1 M stock)
CaCl ₂	0.2 mM	90 µg (or 1.2 ml of 0.5 M stock)

10 Vitamin stock	120 µl
Amino acid dropout mix	6 g
Lysine	330 ml of 100x stock
Adenine	0.9 mM 30 ml of 100x stock

→ bring to pH 6.5 (or another pH) with HCl, autoclave

15 Glucose	2 %	from 40 % stock
KCl		from 1 M stock
essential amino acids (with the exception of Lys/Ade)		from stocks
Agar		

Buffer and solutions:

TE buffer: Tris/HCl (pH 7.5) 10 mM; EDTA (pH 8.0) 1 mM;

TAE buffer: Tris 40 mM; EDTA 1 mM; acetic acid 0.2 mM;

SSC buffer (20x): NaCl 3 M; sodium citrate*2 H₂O 0.3 M;

5 Gel loading buffer: Bromphenol Blue 0.05% (w/v); sucrose 40% (w/v); EDTA, pH 8.0 0.1 M; SDS 0.5% (w/v);

Hybridization buffer: SSC 5x; SDS 0.1% (w/v); dextran sulfate 5% (w/v); stop reagent 1:20;

Buffer A (sterile): Tris-HCl 100 mM; NaCl, pH 9.5 300 mM;

10 Depurination solution: HCl 0.25 M;

Denaturation solution: NaCl 1.5 M; NaOH 0.5 M;

Neutralization solution: NaCl 1.5 M; Tris, pH 8.0 0.5 M.

15 Oligonucleotides (PCR primers):

Name	Sequence (5'→ 3')	RE
TRK1-FL-BamHI-Fo	SEQ ID NO. 7: GCG'GATCCATGCATTTTAGAAGAACGATGAGTAG	BamHI
TRK1-FL-PstI-Re	SEQ ID NO. 8: AGGTTCTGCTGCA'GTTGGTGT	PstI
TRK1-FL-PstI-Fo	SEQ ID NO. 9: ACACCAACTGCA'GCAGAACCT	PstI
TRK1-FL-XhoI-Re	SEQ ID NO. 10: CGC'TCGAGTTAGAGCGTTGTGCTGCTCCT	XhoI
TRK1-Dia-Fo	SEQ ID NO. 11: CCTTACCATTAGCATCACTGAT	---
TRK1-Dia-Re1	SEQ ID NO. 12: CTATTAACCATTTCTCCGCTG	---
URA-Rev	SEQ ID NO. 13: GATTTATCTTCGTTTCCTGCAGGT	---
TRK2-DEL-5-Fo-B	SEQ ID NO. 14: CAC'GTACGTCCAGCACAAATTCACAACAGCT	BsWI
TRK2-DEL-5-Re	SEQ ID NO. 15: CAG'TCGACCTGGATGACGTCCTCTTAGCTG	SaI
TRK2-DEL-3-Fo	SEQ ID NO. 16: CAGAT'ATCATGCTGCCAAGTGACAAACTG	EcoRV
TRK2-DEL-3-Re	SEQ ID NO. 17: TCA'CTAGTTGTTGATGGCTTTGGTTGGT	SpeI
TRK2-Dia-Fo	SEQ ID NO. 18:	---

	GCGAAGAATAGGATGAGATGTG	
TRK2-Dia-Re1	SEQ ID NO. 19: TTGTCGTGGGTCTTCTCTGG	---
KAN-Rev	SEQ ID NO. 20: GCTACCTTTGCCATGTTTCAGAA	---
TOK1-DEL-5-Fo	SEQ ID NO. 21: CAC'GTACGGCAAATTTATCGAGACTCTGCGA	<i>BsWI</i>
TOK1-DEL-5-Re	SEQ ID NO. 22: AGG'TCGACCATATTGCCATATCCCAGCGT	<i>SaI</i>
TOK1-DEL-3-Fo	SEQ ID NO. 23: TGGAT'ATCACCTGATACGCCC	<i>EcoR</i> <i>V</i>
TOK1-DEL-3-Re	SEQ ID NO. 24: CAA'CTAGTGCATACCAGTAGTATGAGACATGCTTG	<i>SpeI</i>
TOK1-Dia-Fo	SEQ ID NO. 25: CCTGAGTACTCAGTACCATCTTG	---
TOK1-Dia-Re1	SEQ ID NO. 26: CTGTAGATGCTGGGCATG	---
Kv1.5-GFP-Fo	SEQ ID NO. 27: TACG'TCGACATGGAGATCGCCCTGGTG	<i>Sall</i>
Kv1.5-GFP-Re	SEQ ID NO. 28: TACG'TCGACATCTGTTTCCCGGCTGGTG	<i>Sall</i>
HERG1-GFP-Fo	SEQ ID NO. 29: TACAT'CGATATGCCGGTGCGGAGGG	<i>Clal</i>
HERG1-GFP-Re	SEQ ID NO. 30: TACG'TCGACACTGCCCGGGTCCGA	<i>Sall</i>

Vectors:

5 Bacterial vectors

Name	Size (bp)	Genes
pcDNA3 (Invitrogen)	5446	CMV prom., T7 prom., polylinker, Sp6 prom., BGH poly (A), SV40 prom., SV 40 ori, Neomycin ^R , SV 40 poly (A), ColE1 ori, Amp ^R
pcDNA3.1 (+/-) (Invitrogen)	5432	CMV prom., T7 prom./priming site, MCS, pcDNA3.1 reverse priming site, BGH poly (A), F1 ori, SV40 prom., SV 40 ori, Neomycin ^R , SV 40 poly (A), ColE1 ori, Amp ^R

pUG6	4009	<i>loxP</i> -TEF2 prom.-kanMX- <i>loxP</i> -TEF2 term., ori, Amp ^R
pCR®-Blunt II-TOPO	3519	<i>lac</i> prom./op., M13 reverse prim. site, LacZ- α ORF, SP6 prom. prim. site, MCS, TOPO™ cloning site, T7 prom. prim. site, M13 (-20) forward prim. site, M13 (-40) prim. site, fusion point, <i>ccdB</i> lethal gene ORF, <i>kan</i> gene, (<i>kan</i> prom., kanamycin resistance gene ORF), zeocin resistance ORF, pMB1 origin (pUC-derived)
pCR® II-TOPO	3900	LacZ- α gene, M13 reverse prim. site, SP6 prom., MCS, T7 prom., M13 (-20) forward prim. site, M13 (-40) forward prim. site, f1 origin, kanamycin resistance ORF, ampicillin resistance ORF, pMB1 origin (pUC-derived)

Yeast vectors

Name	Size(bp)	Genes
pSH47	6786	CEN6/ARSH4, URA3, CYC1 term., CRE, GAL1 prom., Amp
p414 GAL1	5474	CEN6/ARSH4, TRP1, CYC1 term., GAL1 prom., Amp ^R
p416 GAL1	5584	CEN6/ARSH4, URA3, CYC1 term., GAL1 prom., Amp ^R
p416 ADH	6624	CEN6/ARSH4, URA3, CYC1 term., ADH prom., Amp ^R
p423 GPD3	6678	2 μ , HIS3, CYC1 term., GPD3 prom., Amp ^R
p426 GAL1	6417	2 μ , URA3, CYC1 term., GAL1 prom., Amp ^R
p426 GAL1-yEGFP3	7140	2 μ , URA 3, CYC1 term., yEGFP3, GAL1 prom., Amp ^R
p426 GAL1-SP-yEGFP3	7227	2 μ , URA 3, CYC1 term., N-terminal 24 aa of Ste2, yEGFP3, GAL1 prom., Amp ^R

Strains:

5 Bacterial strains: DH5 α ; One Shot™ TOP10 (*Invitrogen*)

Yeast strains:

All yeast strains generated for this work are based on the diploid wild-type strain:

W303 MAT α/a ade2, his3-11-15, leu2-3-112, trp1-1, ura3-1, can1-100;

10 ATCC No. 208352.

Strain	Original Name	Mating type	Genes
YM 96	w303	MAT α/a	ade2, his3-11-15, leu2-3-112, trp1-1, ura3-1, can1-100
YM 97	w303	MAT α	ade2, his3-11-15, leu2-3-112, trp1-1, ura3-1, can1-100
YM 98	w303	MAT α	ade2, his3-11-15, leu2-3-112, trp1-1, ura3-1, can1-100

The following yeast strains were generated:

Strain	Original Name	Mating type	Genes (with the exception of ade2, his3-11-15, leu2-3-112, trp1-1, ura3-1, can1-100)
YM 123	Δ trk1 in YM 96	MAT α	trk1:: <i>hisG</i> -URA3- <i>hisG</i>
YM 124	Δ trk1 in YM 96	MAT α	trk1:: <i>hisG</i> -URA3- <i>hisG</i>
YM 139	Δ tok1 in YM 96	MAT α/a	tok1:: <i>loxP</i> -KanMX- <i>loxP</i>
YM140	Δ tok1 in YM 123	MAT α	trk1:: <i>hisG</i> -URA3- <i>hisG</i> , tok1:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 141	Δ tok1 in YM 123	MAT α	trk1:: <i>hisG</i> -URA3- <i>hisG</i> , tok1:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 142	Δ tok1 in YM 96	MAT α/a	tok1:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 143	Δ tok1 in YM 124	MAT α	trk1:: <i>hisG</i> -URA3- <i>hisG</i> , tok1:: <i>loxP</i> -KanMX- <i>loxP</i>
YM144	Δ tok1 in YM 124	MAT α	trk1:: <i>hisG</i> -URA3- <i>hisG</i> , tok1:: <i>loxP</i> -KanMX- <i>loxP</i>

YM 154	Δ tok1 in YM 96	MAT α	tok1:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 155	Δ tok1 in YM 96	MATa	tok1:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 156	Δ tok1 in YM 96	MATa	tok1:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 157	Δ tok1 in YM 96	MAT α	tok1:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 158	Δ trk2 in YM 96	MAT α	trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 159	Δ trk2 in YM 96	MATa	trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 160	Δ trk2 in YM 96	MATa	trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 161	Δ trk2 in YM 96	MAT α	trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 162	Δ tok1 in YM 123	MAT α	trk1:: <i>hisG</i> , tok1:: <i>loxP</i>
YM 163	Δ tok1 in YM 123	MAT α	trk1:: <i>hisG</i> , tok1:: <i>loxP</i>
YM 164	Δ tok1 in YM 124	MATa	trk1:: <i>hisG</i> , tok1:: <i>loxP</i>
YM 165	Δ tok1 in YM 124	MATa	trk1:: <i>hisG</i> , tok1:: <i>loxP</i>
YM 166	YM 124 x YM 160	MATa	trk1:: <i>hisG</i> -URA3- <i>hisG</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 167	YM 124 x YM 160	MATa	trk1:: <i>hisG</i> -URA3- <i>hisG</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 168	YM 124 x YM 160	MAT α	trk1:: <i>hisG</i> -URA3- <i>hisG</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 169	YM 124 x YM 160	MAT α	trk1:: <i>hisG</i> -URA3- <i>hisG</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 182	Δ trk2 in YM 165	MATa	trk1:: <i>hisG</i> , tok1:: <i>loxP</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 183	YM 166	MATa	trk1:: <i>hisG</i> , tok1:: <i>loxP</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 184	YM 168	MATa	trk1:: <i>hisG</i> , tok1:: <i>loxP</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 185	Kv1.5-pRS426- Gal1-yEGFP3 in YM 97	MATa	pRS426-GAL1 with Kv1.5-GFP3, trk1:: <i>hisG</i> , tok1:: <i>loxP</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 186	Kv1.5-pRS426-	MATa	pRS426-GAL1 with N24 Ste2-Kv1.5-GFP3,

	Gal1-SP-yEGFP3 in YM 97		trk1:: <i>hisG</i> , tok1:: <i>loxP</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 187	Kv1.5-pRS426-Gal1-yEGFP3 in YM 182	MATa	pRS426-GAL1 with Kv1.5-GFP3, trk1:: <i>hisG</i> , tok1:: <i>loxP</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 188	Kv1.5-pRS426-Gal1-SP-yEGFP3 in YM 182	MATa	pRS426-GAL1 with N24 Ste2-Kv1.5-GFP3, trk1:: <i>hisG</i> , tok1:: <i>loxP</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 189	p423-GPD3 in YM 168	MATa	p423-GPD3, trk1:: <i>hisG</i> -URA3- <i>hisG</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 190	Kv1.5-p423-GPD3 in YM 168	MATa	p423-GPD3 with Kv1.5, trk1:: <i>hisG</i> -URA3- <i>hisG</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 191	HERG-p423-GPD3 in YM 168	MATa	p423-GPD3 with HERG, trk1:: <i>hisG</i> -URA3- <i>hisG</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 192	HCN2-p423-GPD3 in YM 168	MATa	p423-GPD3 with HCN2, trk1:: <i>hisG</i> -URA3- <i>hisG</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 193	IRK1-p423-GPD3 in YM 168	MATa	p423-GPD3 with IRK1, trk1:: <i>hisG</i> -URA3- <i>hisG</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 194	p423-GPD3 in YM 182	MATa	p423-GPD3, trk1:: <i>hisG</i> , tok1:: <i>loxP</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 195	Kv1.5-p423-GPD3 in YM 182	MATa	p423-GPD3 with Kv1.5, trk1:: <i>hisG</i> , tok1:: <i>loxP</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 196	HERG-p423-GPD3 in YM 182	MATa	p423-GPD3 with HERG, trk1:: <i>hisG</i> , tok1:: <i>loxP</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>

YM 197	HCN2-p423-GPD3 in YM 182	MATa	p423-GPD3 with HCN2, trk1:: <i>hisG</i> , tok1:: <i>loxP</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 198	IRK1-p423-GPD3 in YM 182	MATa	p423-GPD3 with IRK1, trk1:: <i>hisG</i> , tok1:: <i>loxP</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>
YM 199	TRK1-p423-GPD3 in YM 182	MATa	p423-GPD3 with TRK1, trk1:: <i>hisG</i> , tok1:: <i>loxP</i> , trk2:: <i>loxP</i> -KanMX- <i>loxP</i>

Cloned potassium channels:

A)

5	Systematic name	KCNA5
	Synonyms	Kv1.5, (HK2, HPCN1)
	Family	voltage-gated potassium channel, shaker-related subfamily (member No. 5), delayed rectifier
	Chromosomal localization	12p13.32-p13.31
10	Accession	NID g4504818
	Protein	613 aa, 67 kD
	Distribution in the tissue	heart, pancreatic islets and insulinoma
	Homologs	mKcna5 (<i>Mus musculus</i>), 70% with hHCN4
	References	(Roberds, S. L. et al., 1991; Curran, M. E. et al., 1992; Snyder, D. J. et al., 1993)
15		

B)

	Systematic name	HCN2
	Synonyms	BCNG2 (brain cyclic nucleotide gated channel), HAC1
20	Family	hyperpolarization-activated and cyclic nucleotide gate potassium channel, belongs to the superfamily of the voltage-gated potassium channels
	Chromosomal localization	19p13.3

Accession	NID g4996893 g4775348
Protein	889 aa
Function	pacemaker
Distribution in the tissue	brain, heart
5 Homologs	mHcn2 (<i>Mus musculus</i>)
References	(Ludwig, A. et al., 1999)

C)

Systematic name	KCNH2
10 Synonyms	HERG1 (longer splice variant)
Family	voltage-gated potassium channel, eag related subfamily, member No. 2
Chromosomal localization	7q35-q36
Accession	NID g4557728 g4156210
15 Properties	channel activation by K ⁺ channel regulator 1 accelerated
References	(Taglialatela, M. et al., 1998; Itoh, T. et al., 1998)

D)

Systematic name	KCNJ2 (guinea pig)
20 Synonyms	Kir2.1, IRK1
Family	inwardly rectifying potassium channel
Occurrence in the tissue	brain, heart, lung, kidney, placenta, skeletal musculature
References	(Tang, W. et al., 1995)

25 Methods:

ROMK2 (see appendix "Sequence ROMK2")

PCR:

30 Protocol for Powerscript polymerase (*PAN Biotech*):

Mix for lower reagent (hotstart protocol) (25 μ l):

3 μ l H₂O; 2.5 μ l 10x OptiPerform™ III buffer, pH 9.2; 10 μ l 1.25 mM dNTPs
(= 200 μ M);

1.5 μ l forward primer (20 pmol/ μ l); 1.5 μ l reverse primer (20 pmol/ μ l); 1.5 μ l 50 mM
MgCl₂ (= 1.25 mM); 5 μ l 5x OptiZyme™ enhancer.

5 Mix for upper reagent (35 μ l):

23 μ l H₂O; 3.5 μ l 10x OptiPerform™ III buffer; 1.5 μ l 50 mM MgCl₂; 0.5 μ l
PowerScript DNA polymerase; 7 μ l 5x OptiZyme™ enhancer .

PCR program (hotstart):

- 10 1. 1 min at 94°C
2. 1 min at 94°C
3. 1.5 minutes at 50-55°C (depending on primer)
4. 4 minutes at 69-72°C (depending on polymerase)
5. Repeat 27x from 2.
- 15 6. 4°C ∞
7. End.

Protocol for AmpliTaq polymerase (*Perkin Elmer*):

Mix for upper reagent (hotstart protocol) (50 μ l):

- 20 18.1 μ l H₂O; 4.2 μ l 10x buffer II; 16.7 μ l dNTPs; 2.5 μ l forward primer; 2.5 μ l
reverse primer; 6 μ l 25 mM MgCl₂ (= 1.5 mM).

Mix for lower reagent (50 μ l):

42 μ l H₂O; 5 μ l 10x buffer II; 1 μ l AmpliTaq polymerase; 2 μ l template.

25 DNA purification

Purification of PCR reactions: The purification of PCR amplification products was
carried out using the High Pure PCR Product Purification Kit (Roche)

Phenol extraction: Make up sample volume to 200 μ l with TE buffer. Add 200 μ l of phenol/chloroform/isoamyl alcohol (25:24:1), mix and spin for 1 minute at maximum speed. Transfer top phase into new Eppendorf tube, add 200 μ l of chloroform/isoamyl alcohol, mix, spin for 1 minute. Remove top phase, then precipitate with ethanol.

- 5 Ethanol precipitation: To a sample volume of approx. 200 μ l pipette 5 μ l 5 M NaCl and 20 μ l 3 M NaAc (pH 5.7). Add 2.5 volumes of 100% ethanol, mix, store for at least 30 minutes or longer at -20°C, spin for 10 minutes at 4°C, wash the pellet in 170 μ l of 70% cold ethanol, spin for 3 minutes, and dry pellet at 37°C and resuspend in 30 μ l of H₂O.

- 10 Isolation of plasmid DNA from *E. coli*: The isolation of plasmid DNA from *E. coli* overnight cultures was carried out using the *QIAprep Spin Miniprep Kit Protocol* (Qiagen)

DNA preparation from *Saccharomyces cerevisiae*:

- 15 Incubate the yeast cells overnight at 30°C in 10 ml of YPD, in the morning: spin for 10 minutes at 3000 rpm, and resuspend pellet in 500 μ l of 1 M sorbitol, 0.1 M EDTA (pH 7.5), and transfer into an Eppendorf tube. Add 50 μ l of Zymolase (5 mg/ml, in sorbitol/EDTA), incubate for 1 hour at 37°C and spin for 1 minute. Resuspend the pellet in 500 μ l 50 mM Tris, 20 mM EDTA (pH 7.4). Add 50 μ l 10% SDS, mix thoroughly and incubate for 30 minutes at 65°C, add 200 μ l 5 M KAc, place on ice for 1 hour and spin for 10 minutes. Transfer the supernatant (approx. 650 μ l) into a new Eppendorf tube, add 1 volume of isopropanol, mix gently and leave to stand for 5 minutes. Either spin down briefly or extract precipitated DNA with a glass hook and dry the pellet in the air. Resuspend the pellet or the DNA in 150 μ l of TE buffer and
- 25 dissolve for 10 minutes at 65°C.

DNA cloning techniques: All DNA cloning techniques were carried out following standard protocols.

- 30 Yeast transformation (lithium acetate method):

Incubate the yeast strain to be transformed overnight at 30°C on the shaker in 5 ml of suitable medium; in the morning dilute the overnight culture with suitable medium

(OD₆₀₀ = 0.4-0.5) and incubate for a further 2 hours on the shaker at 30°C (OD₆₀₀ = 0.4-0.8). Spin for 3 minutes at 2500 rpm, wash pellet with 25 ml of sterile H₂O, spin for 3 minutes at 2500 rpm; resuspend pellet in 1 ml of LITE (100 mM LiAc, TE pH 7.5) and transfer suspension into an Eppendorf tube. Incubate for 5 minutes at RT, spin for 5 15 sec (Quickspin); wash pellet with 1 ml of 100 mM LiAc, quick-spin; depending on the cell density, resuspend pellet in 200-400 µl of 100 mM LiAc and divide into 50 µl aliquots.

Add the following in the exact sequence stated:

- 240 µl PEG (50%), mix suspension by gently pipetting
- 10 36 µl 1 M LiAc, mix suspension by gently pipetting
 - 10 µl ss-sperm DNA (stored at -20°C; prior to use, heat for 10 minutes at 80-90°C, then transfer to ice)
 - 2-3 µg plasmid DNA (or 8-10 µl of Miniprep in the case of knock-out transformation), mix suspension by gently pipetting
- 15 Incubate transformation reaction for 30 minutes at 30°C in an overhead rotator at slow speed
 - Transformation reaction for 15 minutes at 42°C
 - Quick-spin, resuspend pellet in 200 µl of TE buffer (in the case of knock-out: resuspend pellet in 300 µl of YPD and incubate in an overhead rotator for 4 hours at
- 20 30°C
 - Plate 100 µl per agar plate (in the case of knock-out of all of the reaction) and incubate for 3-4 days at 30°C

Sequencing: ABI PRISM™red. protokoll/AmpliTa[®]q FS ¼ BigDyeTerminator

Reaction:

Premix	2 μ l
DNA template	
ss DNA	50 ng
ds DNA	250 ng
PCR products (0.2-5 kB)	10 – 50 ng
Primer	3-10 pmol
H ₂ O to final volume	10 μ l

Thermocycler protocol (25 cycles):

1. 15 seconds at 96°C
- 5 2. 15 seconds at 96°C
3. 10 seconds at 55°C
4. 4 minutes at 60°C
5. return to 2., 24x
6. 4°C ∞
- 10 7. End.

Purification reaction (Centri Sep Spin Columns, *Princeton Separations*):

Pre-soak column with 750 μ l of H₂O for 30 minutes; drain liquid; spin for 2 minutes at 3000 rpm; make up reaction to 20 μ l with H₂O and apply to column; spin for 2 minutes
15 at 3000 rpm.

Sample application: in sequencing tubes, 4 μ l of Centri Sep eluate + 20 μ l of TSR (template suppression reagent); denature for 2 minutes at 90°C.

Southern Blot:

20

Digest DNA probe with suitable restriction enzymes, separate by gel electrophoresis and extract from the gel. Digest genomic DNA overnight with suitable restriction enzymes and separate by gel electrophoresis (1% agarose gel)

Pretreatment of the gel: Remove loading wells from the agarose gel. Depurinate the agarose gel for 15 minutes in 0.25 M HCl, then wash 2x in distilled water; denature the agarose gel for 30 minutes in 0.5 M NaOH; transfer using the Vacuum Blotter Model 785 (*BioRad*): into the center of the vinyl sheet, cut a window (window seal), trim the edges of the nylon membrane and the filter paper in each case 0.5 cm smaller than the gel, moisten the edge of the nylon membrane with distilled water in each case 0.5 cm wider than the window in the vinyl sheet, then moisten nylon membrane and filter paper with transfer solution

Construction of the apparatus (bottom to top):

- 10 Base unit, vacuum platform, porous vacuum slab, filter paper, nylon membrane, vinyl window, agarose gel, final frame, lid

Preheat BioRad vacuum pump for 10 minutes, apply vacuum (5 inches Hg)

Press gel gently along the edge

Place transfer solution (approx. 1 l 10x SSC) into upper reservoir; transfer time:

- 15 90 minutes; switch off vacuum, remove nylon membrane and rinse for 5 minutes in 2x SSC, then leave to dry in the air between filter paper. DNA immobilization: place nylon membrane on UV-permeable cling-film and apply probe at the edge as positive control; place into the UV stratalinker and start crosslinking (1200000 J \rightarrow 0); membrane may be stored in cling-film or between Whatman filter paper at room temperature or 4°C.

- 20 *Gene Images* Random Prime Labelling Module (*Amersham*):

Labeling of the DNA probe: Denature DNA probe for 5 minutes at 96°C (heat shock), then place on ice. 10 μ l reaction mix (nucleotide mix (5x), fluorescein-11-dUTP, dATP, dCTP, dGTP and dTTP in Tris-HCl, pH 7.8, 2-mercaptoethanol and $MgCl_2$); 5 μ l of primer (Random Nonamers); 1 μ l of enzyme solution (Klenow fragment, 5 units/ml); 22

- 25 μ l of denatured DNA probe; 12 μ l of H_2O . Incubate for 2 hours at 37°C and add 2 μ l of 0.5 M EDTA (=20 mM), store aliquots at -20°C. Verification of the labeling efficiency: dilute 5x nucleotide mix with TE buffer 1/5, 1/10, 1/25, 1/50, 1/100, 1/250 and 1/500; to a nylon membrane strip, apply 5 μ l of DNA probe together with 5 μ l of 1/5 dilution, allow to absorb briefly and wash for 15 minutes at 60°C in prewarmed 2x SSC; apply
- 30 to a reference membrane strip the remaining solutions without the 1/5 dilution and observe both membrane strips under UV light \rightarrow determination of the sample intensity.

Hybridization: Prehybridize nylon membrane (blot) with warmed hybridization buffer (0.3 ml/cm²) for 2 hours at 60°C in a rotating oven; drain buffer and retain 10 ml thereof; denature DNA probe (20 µl); (5 minutes at 96°C, then cool on ice); place probe with the 10 ml of buffer onto blot and hybridize overnight at 60°C in the rotating oven.

5

Wash steps:

15 minutes on platform shaker in warmed 1x SSC, 0.1% (w/v) SDS; 15 minutes on a platform shaker in warmed 0.5x SSC, 0.1% (w/v) SDS

10 Gene Images CDP-*Star* Detection Module (Amersham):

Stop and antibody reaction: On a shaker, incubate the blot at room temperature for 1 hour in a 1/10 dilution of stop reagent in buffer A; dilute antibody solution (alkaline phosphatase coupled to antiluorescein, 5000x) with 0.5% (w/v) BSA/buffer A, together

15 with the blot seal into foil and incubate for 1 hour at room temperature on a shaker; remove unbound antibody solution by washing three times for 10 minutes in 0.3% Tween 10 in buffer A

Signal generation and detection: Drain wash buffer, place blot on cling-film; apply 5 ml of detection reagent, allow to react for 2-5 minutes and again drain (the alkaline
20 phosphatase causes the generation of light); wrap in cling-film and, in a dark room in red light, apply the film (Hyperfilm™ MP, *Amersham*), expose for 0.5 2 hours in a film cassette (BioMax, *Kodak*), develop and scan; the blot can be stored in cling-film at 4°C.

25 Example 1: Construction of the specific deletion cassettes

All deletions were carried out by standard methods (Fink, G. R. et al., 1991; Wach, A. et al., 1994; Guldener, U. et al., 1996; Goldstein, A. L. et al., 1999).

Fragments of about 500 bp each, each of which represents the region at the beginning
30 and the end of the gene, was amplified by PCR with the primers TRK1-FL-BamHI-Fo, TRK1-FL-PstI-Re, TRK1-FL-PstI-Fo and TRK1-FL-XhoI-Re for TRK1 or TRK2-DEL-5-

Fo-B, TRK2-DEL-5-Re, TRK2-DEL-3-Fo and TRK2-DEL-3-Re for TRK2 and TOK1-DEL-5-Fo, TOK1-DEL-5-Re, TOK1-DEL-3-Fo and TOK1-DEL-3-Re for TOK1 (see Chapter 2.3). The amplified termini later allow correct integration into the yeast genome. The yeast strain *w303 a/a* or *w303 a/a Δ trk1* acted as DNA template.

5

Example 2: Construction of the single, double and triple mutants

Example 2 a: Single knock-out

The constructed deletion cassettes for TRK1, TRK2 and TOK1 were each transformed into the diploid yeast strain YM 96 (MATa/MATα). Integration of the deletion cassettes to the genome was verified by growing the *trk1* mutants (YM123/124) on (-)URA/Glc and the *trk2*- (YM158-161) and *tok1* mutants (YM154-157) on YPD/geneticin, since the URA3 marker in the TRK1 deletion cassette allows growth on (-)URA medium and the KAN marker in the TRK2 or TRK1 deletion cassette allows growth on geneticin (Fink, G. R. et al., 1991). The positive colonies were transferred to a sporulation plate by replica plating, whereupon MATa/MATα diploid cells sporulate after 18-24 hours without vegetative growth. After they were treated with Zymolase and regrown on YPD, tetrads of some colonies were then divided into 4 individual spores with the aid of a dissecting microscope.

The mating type of the spore colonies was determined by pairing with matching tester strains (Fink, G. R. et al., 1991). Selection for the presence of the deletion cassette was done by replica-plating on -URA medium (for *trk1*) and on geneticin-containing medium for *trk2* and *tok1*. After obtaining the genomic DNA of the transformants by yeast DNA preparation, the result was verified by diagnostic PCR and Southern blot.

25

Example 2 b: Double knock-out

The TOK1 deletion cassette was transformed into the haploid *Δtrk1* yeast strains YM123 and Y124 and selected for integration of the TOK1 deletion cassette by growth on YPD/geneticin. The result was verified by diagnostic PCR and Southern blot. Glycerol cultures were made with the (+)URA3,(+)KAN (*Δtrk1 Δtok1*) strains (YM140, YM141, YM143 and YM144).

Single colonies were streaked out as patches, replica-plated on 5-FOA, and colonies were selected which had eliminated the URA3 marker and a *hisG* repeat from the TRK1 deletion cassette (Fink, G. R. et al., 1991). Accordingly, no colonies which lacked the URA3 gene (in TRK1) for uracil synthesis grew on (-)URA/Glc, while all 5 colonies survived on YPD/gen owing to the resistance gene in the TOK1 deletion cassette. To remove the Kan marker from the genome, the (-)URA3 mutants were transformed with plasmid pSH47, on which the genes for Cre recombinase and uracil synthesis (URA3) are located. Positive transformants grew on (-)URA/Glc and it was then possible to induce Cre recombinase by incubation in (-)URA/Gal liquid medium. In 10 this process, the Kan marker together with one *loxP* repeat is eliminated, and one *loxP* remains.

After the overnight culture was brought to OD₆₀₀ = 5, the dilutions 1:10 000 and 1:50 000 were plated onto (-)URA/Gal. Patches of single colonies, replica-plated on YPD/gen, showed no growth (this means that the Kan marker had been eliminated 15 successfully). To remove plasmid pSH47, the cells were subsequently reselected twice 5-FOA. Glycerol cultures were made with the (-)URA(-)KAN ($\Delta trk1 \Delta tok1$) strains (YM162, YM163 and YM164).

Example 2 c: Triple knock-out

20

Overnight cultures in YPD were set up with single $\Delta trk1 \Delta tok1$ single colonies (YM162 and YM164), and, next day, transformed with the *BsWI*/*SpeI*-digested TRK2 deletion cassette and plated onto YPD/KCl/geneticin. After a yeast DNA preparation, the triple knock-out was verified by diagnostic PCR and Southern blot.

25

Table 1

Top row, left to right:	Bottom row, left to right:
1. marker	1. marker
2. YM 97 with TRK1 DiaFo/Re1	2. YM 182 with TRK1 DiaFo/Re1
3. YM 97 with TRK2 DiaFo/Re1	3. YM 182 with TRK2 DiaFo/Re1
4. YM 97 with TOK1 DiaFo/Re1	4. YM 182 with TOK1 DiaFo/Re1

5. YM 97 with TRK1 DiaFo/URARe	5. YM 182 with TRK1 DiaFo/URARe
6. YM 97 with TRK2 DiaFo/KANRe	6. YM 182 with TRK2 DiaFo/KANRe
7. YM 97 with TOK1 DiaFo/KANRe	7. YM 182 with TOK1 DiaFo/KANRe
8. free	8. free
9. YM 97 with TRK1 DiaFo/Re2	9. YM 182 with TRK1 DiaFo/Re2
10. YM 97 with TRK2 DiaFo/Re2	10. YM 182 with TRK2 DiaFo/Re2
11. YM 97 with TOK1 DiaFo/Re2	11. YM 182 with TOK1 DiaFo/Re2

Example 3: Subcloning and transformation of the human potassium channels into the double and triple mutants

5 The human genes HERG, HCN2, Kv1.5 and, as positive controls, TRK1 and IRK1 (guinea pig) were excised from the plasmids harboring them (HERG between *Bam*HI in pcDNA; HCN2 between *Nco*I/*Xho*I in pTLN; Kv1.5 between *Nhe*I/*Eco*RI in pcDNA3.1(-); IRK1 between *Bam*HI/*Eco*RI in pSGEM) by cleavage with restriction enzymes, separated by gel electrophoresis and extracted from the gel. The individual

10 human potassium channels were ligated into the yeast vector p423-GPD3 (Mumberg, D. et al., 1995; Ronicke, V. et al., 1997) and transformed into *E. coli*. Control digestion of the plasmid preparations and sequencing permitted the identification of the clones which had integrated the human gene. The plasmids were subsequently transformed into the $\Delta trk1 \Delta trk2$ double knock-out (YM 168) and into the $\Delta trk1 \Delta trk2 \Delta tok1$ triple

15 knock-out (YM 182) and plated onto (-)HIS/80 mM KCl.

Example 4: Characterization of the knock-out strains

Example 4 a: Growth of the double and triple mutants on culture plates at various K⁺

20 concentrations and pH values

To compare the different potassium requirements of the various knockouts, yeast strains YM 182, YM 168 and YM 97 (WT) were incubated on DPM plates with different K⁺ concentrations and different pH values. To this end, patches of the glycerol cultures

were first streaked onto 100 mM KCl/pH 6.5. After 2 days' growth, 50 mM, 30 mM and 5 mM KCl were replica-plated.

This experiment showed that both strain YM168 ($\Delta trk1 \Delta trk2$) and strain YM182 ($\Delta trk1$
 5 $\Delta trk2 \Delta tok1$) are viable on 50 mM and 30 mM KCl. Additionally, it emerged that strain YM182 grew better in the presence of 30 mM KCl than strain YM168. None of the two strains was viable in the presence of 5 mM KCl, in contrast to the wild-type strain YM97.

To test for pH-dependency, the three strains were additionally replica-plated on
 10 100 mM and 5 mM KCl/pH 5.0 and on 100 mM and 5 mM KCl/pH 4.0. This experiment demonstrated that neither YM168 nor YM182 are viable at pH 4.0 in the presence of 100 mM KCl and 5 mM KCl. At pH 5.0 and 100 mM KCl, the growth deficiency of YM168 is more pronounced than in the case of strain YM182. Expression of TRK1 of vector pRS416GAL1 fully compensates for the growth deficiency of strains YM168
 15 ($\Delta trk1 \Delta trk2$) and YM182 ($\Delta trk1 \Delta trk2 \Delta tok1$).

Example 4 b: Growth of double and triple mutants in liquid medium at various K^+ concentrations

20 To characterize strains YM168 ($\Delta trk1 \Delta trk2$) and YM182 ($\Delta trk1 \Delta trk2 \Delta tok1$), on which all further experiments are based, the growth behavior of the yeast strains in liquid culture was studied. First, overnight cultures were set up in DPM/80 mM KCl, and, next morning, the cultures were brought to an OD = 0.05 with DPM/5 mM KCl and with DPM/15 mM KCl. The optical density at 600 nm was determined after defined intervals
 25 with the aid of a photometer.

These studies demonstrate that the growth deficiency of strain YM182 is less pronounced at 5 mM KCl and at 15 mM KCl than in the case of strain YM168.

30 Example 5: Characterization of the human potassium channels in double and triple knock-outs

Example 5 a: Complementation capacity for K^+ deficiency on culture plates

Each of the strains YM168 ($\Delta trk1 \Delta trk2$) and YM182 ($\Delta trk1 \Delta trk2 \Delta tok1$) was transformed with the human potassium channels Kv1.5 ((Fedida, D. et al., 1998); YM190 and YM195) and HERG1 ((Fedida, D. et al., 1998) ; YM191 and YM196) in p423-GPD3, respectively, as yeast expression vector. gplRK1 ((Tang, W. et al., 1995); YM193 and YM198) acted as positive control in p423-GPD3 as yeast expression vector (Mumberg, D. et al., 1995; Ronicke, V. et al., 1997). The blank vector p423-GPD3 (YM189 and YM194) acted as negative control. The transformed yeast strains 10 were plated onto (-)HIS/80 mM KCl medium. After this, patches of single colonies were replica-plated onto DPM/5 mM KCl (pH 6.5) to check the capacity of complementing the potassium deficiency.

These experiments demonstrated that the positive control gplRK1 (YM193 and YM198) in p423-GPD3 fully complemented growth deficiency of double and triple 15 knock-outs. The blank vector p423-GPD3 (YM189 and YM194) as negative control is not capable of complementing the growth deficiency. While the human potassium channel Kv1.5 complements the growth deficiency of triple knock-out, it does so significantly less effectively than the positive control gplRK1. It was also observed that the human potassium channel Kv 1.5 does not complement the double knock-out 20 $\Delta trk1 \Delta trk2$. Under the given experimental conditions, the HERG1 channel does not complement the growth deficiency of double and triple knock-outs.

Example 5 b: Growth on culture plates in the presence of activators

25 To demonstrate the effect of activators on the various potassium channels, the strains stated above were incubated in media containing the following specific activators.
Kv1.5: Rb^+ extends the hyperpolarization phase. This means that the inwardly directed K^+ flux is more prolonged and increases the possibility of complementing the growth deficiency.

HERG: Cs^+ extends the hyperpolarization phase. This means that the inwardly directed K^+ flux is more prolonged and increases the possibility of complementing the growth deficiency. This channel is inhibited by Cs^+ .

IRK1: Cs^+ blocks this channel.

- 5 The experiments with p423-GPD3-Kv1.5 demonstrated that the human Kv1.5 channel is capable of fully complementing the growth deficiency of the $\Delta trk1 \Delta trk2 \Delta tok1$ mutant in the presence of 2 mM RbCl (Fig. 3). Complementation of the growth deficiency of the $\Delta trk1 \Delta trk2$ mutant is markedly less effective (Fig. 3). This tallies with the results shown in Example 6 a.

10

The experiments with p423-GPD3-HERG demonstrated that the human HERG1 channel is capable of fully complementing the growth deficiency of the $\Delta trk1 \Delta trk2 tok1$ mutant in the presence of 2 mM CsCl (Fig. 4). Complementation of the growth deficiency of the $\Delta trk1 \Delta trk2$ mutant is markedly less effective (Fig. 4). This tallies with

- 15 the results shown in Example 6 a.

Example 5 c: Complementation by the Kv1.5 channel in the $\Delta trk1 \Delta trk2 \Delta tok1$ mutant in the presence of RbCl in liquid medium

- 20 The yeast strains YM 194 and YM 195 were tested in DPM/-HIS/5 mM KCl with 1 mM RbCl for the different growth behavior in liquid medium. To this end, 10 ml of overnight culture were set up in DPM/-HIS/80 mM KCl and, next morning, brought to an OD_{600} of 0.05 with the relevant media (final volume: 20 ml). The optical density at 600 nm was determined at defined intervals with the aid of a photometer.
- 25 These experiments demonstrate unambiguously that the expression of Kv1.5 of vector p423-GPD3 in a yeast strain which is deleted for TRK1, TRK2 and TOK1 is capable of complementing the growth deficiency caused thereby.

In further experiments, it was demonstrated that the complementation of the growth
30 deficiency by Kv1.5 and also by gplRK1 is inhibited in the presence of 2 mM CsCl.

Example 5 d: Complementation by the HERG1 channel in the $\Delta trk1 \Delta trk2 \Delta tok1$ mutant in the presence of CsCl in liquid medium

The yeast strains YM 194 and YM 196 were tested in DPM/-HIS/5 mM KCl with 1 mM CsCl for their different growth behavior in liquid medium. To this end, 10 ml of overnight culture were set up in DPM/-HIS/80 mM KCl and, next morning, brought to an OD₆₀₀ of 0.05 with the relevant media (final volume: 20 ml). The optical density at 600 nm was determined at defined intervals with the aid of a photometer.

These experiments demonstrate unambiguously that the expression of HERG1 of vector p423-GPD3 in a yeast strain which is deleted for TRK1, TRK2 and TOK1 is capable of complementing the growth deficiency caused thereby.

Example 6:

15 All growth assays in the triple mutant $\Delta trk1 \Delta trk2 \Delta tok1$ were carried out in growth medium DPM (defined potassium medium) at the pH and the potassium concentration stated in each case.

The substances employed as inhibitors of the human HERG1 K⁺ channel were
 20 terfenadine (α -(4-tert-butylphenyl)-4-(α -hydroxy- α -phenylbenzyl)-1-piperidinebutanol; HMR), pimozone (1-(4,4-bis(P-fluorophenyl)butyl)-4-(2-oxo-1-benzimidazoliny)-piperidine; Sigma, Cat. No. P100), ziprasidone (5-(2-[4-(1,2-benzisothiazol-3-yl)piperazino]-ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one; HMR), loratidine (ethyl 4-(8-chloro-5,6-dihydro-11H-benzo[5,6]cyclohepta[1,2-b]pyridin-11-ylidene)-1-
 25 piperidinecarboxylate; HMR) and sertindole (1-(2-[4-[5-chloro-1-(4-fluorophenyl)-1H-indol-3-yl]-1-piperidinyl]ethyl)-2-imidazolidinone; HMR) (Richelson, E. 1996; Richelson, E. 1999; Delpon, E. et al., 1999; Kobayashi, T. et al., 2000; Drici, M. D. et al., 2000). Diphenhydramine (Sigma, Cat. No. D3630) and fexofenadine (4-[hydroxy-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]butyl]- α,α -dimethyl benzeneacetic acid
 30 hydrochloride; HMR) (Tagliatela, M. et al., 1999; DuBuske, L. M. 1999), substances which should not have inhibitory effect on potassium channels, were also employed.

All substances, dissolved in DMSO, were employed in a final concentration of 30 μ M. As a control, cells were measured with the same final concentration of 0.5% DMSO without substance, without DMSO addition or without substance.

- 5 As described in Figures 1 and 2, the human HERG1 channel is capable of complementing the growth deficiency of the triple mutant $\Delta trk1\Delta trk2\Delta tok1$ on medium which only contains 5 mM KCl. It was possible to demonstrate (Fig. 7, Fig. 8) that, in the presence of the substances terfenadine, pimozone, ziprasidone, sertindole and loratadine, the human HERG1 channel can no longer complement the growth
10 deficiency of the triple mutant $\Delta trk1\Delta trk2\Delta tok1$ on medium which only contains 5 mM KCl.

Example 7:

- 15 Incubation with the substances terfenadine, pimozone, diphenhydramine, ziprasidone, loratidine, fexofenadine and sertindole of the wild-type strain which expresses all three endogenous potassium channel proteins of yeast demonstrated that terfenadine, loratidine and sertindole are specific inhibitors of the human HERG1 channel (Figure 9).

20

According to the present results, pimozone and ziprasidone must be considered as rather unspecific inhibitors. This means that these substances possibly inhibit not only the human HERG1 channel, but also the endogenous potassium channels of the yeast *Saccharomyces cerevisiae*. However, the present results could not exclude that the
25 inhibitory effect found for these substances can possibly also be attributed to an inhibition of other proteins which are essential for the growth of yeast cells. To study this possibility, the action of these substances was also tested in a growth medium containing 80 mM KCl.

- 30 These studies demonstrated (Figure 10) that pimozone inhibits the activity of the essential endogenous potassium channels TRK1 and TRK2 in an unspecific fashion.

The absence of an inhibitory effect of higher potassium concentrations allows the conclusion that pimozide has no generally toxic effect on yeast cells. In contrast, it was demonstrated that ziprasidone inhibits the growth of the yeast cells even at higher potassium concentrations and therefore has a toxic effect on *Saccharomyces cerevisiae*. The identification of the target protein in the yeast which might be responsible for this effect is as yet outstanding.

In conclusion, these experiments demonstrate that the above-described system makes it possible in practice to identify, in the yeast *Saccharomyces cerevisiae*, substances which specifically inhibit the human potassium channels.

10

The results can be seen from Figure 10.

Example 8:

15 The human potassium channels HERG1 and Kv1.5 do not complement the growth deficiency of the double mutant $\Delta trk1\Delta trk2$ (Figure 11 and Figure 12).

Results: Figures 11 and 12.

Figures 11 and 12 demonstrate that the human potassium channels HERG1 and Kv1.5 do not complement the growth deficiency of the double mutant $\Delta trk1\Delta trk2$ (in each case 4th segment in Figures 11 and 12). The comparison with the negative control, i.e. the blank vector $\Delta p423GPD$ in the triple mutant $\Delta trk1\Delta trk2\Delta tok1$ (in each case 1st segment of Figures 11 and 12), shows no improved growth. The negative control $\Delta p423GPD$ in the double mutant $\Delta trk1\Delta trk2$ is not shown, but does not differ from the negative control $\Delta p423GPD$ in the triple mutant $\Delta trk1\Delta trk2\Delta tok1$. In contrast, the human potassium channels HERG1 and Kv1.5 complement the growth deficiency of the triple mutant $\Delta trk1\Delta trk2\Delta tok1$ (in each case 3rd segment of Figures 11 and 12).

Example 9:

30

The human potassium channel ROMK2 ((Shuck, M. E. et al., 1994; Bock, J. H. et al., 1997); Sequence SEQ ID NO. ^{3A}hROMK2) was subcloned into the yeast vector p423GPD and transformed into the triple mutant $\Delta trk1\Delta trk2\Delta tok1$. The studies demonstrated that this human potassium channel too is capable of complementing the growth deficiency of the triple mutant $\Delta trk1\Delta trk2\Delta tok1$.

The capability of this human potassium channel to complement the growth deficiency of the double mutant $\Delta trk1\Delta trk2$ has not been studied as yet. No substances are known as yet which specifically inhibit the ROMK2 channel.

10

The results can be seen from Figure 13.

References

- 15 Curran, M. E., Landes, G. M., and Keating, M. T. Molecular cloning, characterization, and genomic localization of a human potassium channel gene. *Genomics* 12: 729 - 737. (1992)

Dascal, N., Schreibmayer, W., Lim, N. F., Wang, W., Chavkin, C., DiMagno, L., Labarca, C., Kieffer, B. L., Gaveriaux-Ruff, C., and Trollinger, D. Atrial G protein-activated K⁺ channel: expression cloning and molecular properties.

20 *Proc.Natl.Acad.Sci.U.S.A.* 90: 10235 - 10239. (1993)

Fairman, C., Zhou, X., and Kung, C. Potassium uptake through the TOK1 K⁺ channel in the budding yeast. *J.Membr.Biol.* 168: 149 - 157. (1999)

Fedida, D., Chen, F. S., and Zhang, X. The 1997 Stevenson Award Lecture. Cardiac K⁺ channel gating: cloned delayed rectifier mechanisms and drug modulation.

25 *Can.J.Physiol.Pharmacol.* 76: 77 - 89. (1998)

Fink, G. R. and Guthrie, C. Guide to Yeast Genetics and Molecular Biology. Guthrie, C. and Fink, G. R. (1994). 1991. Academic Presss, Inc. Methods in Enzymology.

Ref Type: Book, Whole

Gaber, R. F., Styles, C. A., and Fink, G. R. TRK1 encodes a plasma membrane protein required for high-affinity potassium transport in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 8: 2848 - 2859. (1988)

Goldstein, A. L. and McCusker, J. H. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae* [In Process Citation] *Yeast.* 15: 1541 - 1553. (1999)

Goldstein, S. A., Price, L. A., Rosenthal, D. N., and Pausch, M. H. ORK1, a potassium-selective leak channel with two pore domains cloned from *Drosophila melanogaster* by expression in *Saccharomyces cerevisiae* [published erratum appears in *Proc Natl Acad Sci U S A* 1999 Jan 5;96(1):318] *Proc. Natl. Acad. Sci. U.S.A.* 93: 13256 - 13261. (1996)

Guldener, U., Heck, S., Fielder, T., Beinhauer, J., and Hegemann, J. H. A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res.* 24: 2519 - 2524. (1996)

Ikeda, K., Kobayashi, K., Kobayashi, T., Ichikawa, T., Kumanishi, T., Kishida, H., Yano, R., and Manabe, T. Functional coupling of the nociceptin/orphanin FQ receptor with the G-protein-activated K⁺ (GIRK) channel. *Brain Res. Mol. Brain Res.* 45: 117 - 126. (1997)

Itoh, T., Tanaka, T., Nagai, R., Kamiya, T., Sawayama, T., Nakayama, T., Tomoike, H., Sakurada, H., Yazaki, Y., and Nakamura, Y. Genomic organization and mutational analysis of HERG, a gene responsible for familial long QT syndrome. *Hum. Genet.* 102: 435 - 439. (1998)

Jan, L. Y. and Jan, Y. N. Cloned potassium channels from eukaryotes and prokaryotes. *Annu. Rev. Neurosci.* 20:91-123: 91 - 123. (1997)

Jelacic, T. M., Sims, S. M., and Clapham, D. E. Functional expression and characterization of G-protein-gated inwardly rectifying K⁺ channels containing GIRK3. *J. Membr. Biol.* 169: 123 - 129. (1999)

Ketchum, K. A., Joiner, W. J., Sellers, A. J., Kaczmarek, L. K., and Goldstein, S. A. A new family of outwardly rectifying potassium channel proteins with two pore domains in tandem. *Nature* 376: 690 - 695. (1995)

Ko, C. H., Buckley, A. M., and Gaber, R. F. TRK2 is required for low affinity K⁺ transport in *Saccharomyces cerevisiae*. *Genetics* 125: 305 - 312. (1990)

Ko, C. H. and Gaber, R. F. TRK1 and TRK2 encode structurally related K⁺ transporters in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 11: 4266 - 4273. (1991)

Kubo, Y., Reuveny, E., Slesinger, P. A., Jan, Y. N., and Jan, L. Y. Primary structure and functional expression of a rat G-protein-coupled muscarinic potassium channel [see comments] *Nature* 364: 802 - 806. (1993)

Ludwig, A., Zong, X., Stieber, J., Hullin, R., Hofmann, F., and Biel, M. Two pacemaker channels from human heart with profoundly different activation kinetics. *EMBO J.* 18: 2323 - 2329. (1999)

Madrid, R., Gomez, M. J., Ramos, J., and Rodriguez-Navarro, A. Ectopic potassium uptake in *trk1 trk2* mutants of *Saccharomyces cerevisiae* correlates with a highly hyperpolarized membrane potential. *J. Biol. Chem.* 273: 14838 - 14844. (1998)

Main, M. J., Brown, J., Brown, S., Fraser, N. J., and Foord, S. M. The CGRP receptor can couple via pertussis toxin sensitive and insensitive G proteins. *FEBS Lett.* 441: 6 - 10. (1998)

Mumberg, D., Muller, R., and Funk, M. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* 156: 119 - 122. (1995)

Myers, A. M., Pape, L. K., and Tzagoloff, A. Mitochondrial protein synthesis is required for maintenance of intact mitochondrial genomes in *Saccharomyces cerevisiae*. *EMBO J.* 4: 2087 - 2092. (1985)

Nakamura, R. L., Anderson, J. A., and Gaber, R. F. Determination of key structural requirements of a K⁺ channel pore. *J. Biol. Chem.* 272: 1011 - 1018. (1997)

Roberds, S. L. and Tamkun, M. M. Cloning and tissue-specific expression of five voltage-gated potassium channel cDNAs expressed in rat heart.

Proc.Natl.Acad.Sci.U.S.A. 88: 1798 - 1802. (1991)

Ronicke, V., Graulich, W., Mumberg, D., Muller, R., and Funk, M. Use of conditional
5 promoters for expression of heterologous proteins in *Saccharomyces cerevisiae*.

Methods Enzymol. 283:313-22: 313 - 322. (1997)

Sanguinetti, M. C. and Zou, A. Molecular physiology of cardiac delayed rectifier K⁺
channels. *Heart Vessels Suppl* 12: 170 - 172. (1997)

Schreibmayer, W., Dessauer, C. W., Vorobiov, D., Gilman, A. G., Lester, H. A.,
10 Davidson, N., and Dascal, N. Inhibition of an inwardly rectifying K⁺ channel by G-
protein alpha- subunits. *Nature* 380: 624 - 627. (1996)

Smith, F. W., Ealing, P. M., Hawkesford, M. J., and Clarkson, D. T. Plant members of a
family of sulfate transporters reveal functional subtypes. *Proc.Natl.Acad.Sci.U.S.A.* 92:
9373 - 9377. (1995)

15 Snyder, D. J., Tamkun, M. M., and Bennett, P. B. A rapidly activating and slowly
inactivating potassium channel cloned from human heart. Functional analysis after
stable mammalian cell culture expression. *J.Gen.Physiol.* 101: 513 - 543. (1993)

Taglialatela, M., Castaldo, P., Pannaccione, A., Giorgio, G., and Annunziato, L. Human
ether-a-gogo related gene (HERG) K⁺ channels as pharmacological targets: present
20 and future implications. *Biochem.Pharmacol.* 55: 1741 - 1746. (1998)

Tang, W., Ruknudin, A., Yang, W. P., Shaw, S. Y., Knickerbocker, A., and Kurtz, S.
Functional expression of a vertebrate inwardly rectifying K⁺ channel in yeast.
Mol.Biol.Cell 6: 1231 - 1240. (1995)

Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. New heterologous modules
25 for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast.* 10:
1793 - 1808. (1994)

Wang, Q., Chen, Q., and Towbin, J. A. Genetics, molecular mechanisms and management of long QT syndrome. *Ann.Med.* 30: 58 - 65. (1998)

Wilde, A. A. and Veldkamp, M. W. Ion channels, the QT interval, and arrhythmias. *Pacing.Clin.Electrophysiol.* 20: 2048 - 2051. (1997)

- 5 Wischmeyer, E., Doring, F., Spauschus, A., Thomzig, A., Veh, R., and Karschin, A. Subunit interactions in the assembly of neuronal Kir3.0 inwardly rectifying K⁺ channels. *Mol.Cell Neurosci.* 9: 194 - 206. (1997)

Yamada, M., Inanobe, A., and Kurachi, Y. G protein regulation of potassium ion channels. *Pharmacol.Rev.* 50: 723 - 760. (1998)

- 10 Bock, J. H., Shuck, M. E., Benjamin, C. W., Chee, M., Bienkowski, M. J., and Slightom, J. L. Nucleotide sequence analysis of the human KCNJ1 potassium channel locus *Gene* 188: 9 - 16. (1997)

Delpon, E., Valenzuela, C., and Tamargo, J. Blockade of cardiac potassium and other channels by antihistamines *Drug Saf* 21 Suppl 1:11-8; discussion 81-7: 11 - 18. (1999)

- 15 Drici, M. D. and Barhanin, J. Cardiac K⁺ channels and drug-acquired long QT syndrome *Therapie* 55: 185 - 193. (2000)

DuBuske, L. M. Second-generation antihistamines: the risk of ventricular arrhythmias *Clin.Ther.* 21: 281 - 295. (1999)

- 20 Itoh, T., Tanaka, T., Nagai, R., Kikuchi, K., Ogawa, S., Okada, S., Yamagata, S., Yano, K., Yazaki, Y., and Nakamura, Y. Genomic organization and mutational analysis of KVLQT1, a gene responsible for familial long QT syndrome *Hum.Genet.* 103: 290 - 294. (1998)

- 25 Kobayashi, T., Ikeda, K., and Kumanishi, T. Inhibition by various antipsychotic drugs of the G-protein-activated inwardly rectifying K(+) (GIRK) channels expressed in Xenopus oocytes *Br.J.Pharmacol.* 129: 1716 - 1722. (2000)

Richelson, E. Preclinical pharmacology of neuroleptics: focus on new generation compounds *J.Clin.Psychiatry* 57 Suppl 11:4-11: 4 - 11. (1996)

Richelson, E. Receptor pharmacology of neuroleptics: relation to clinical effects [see comments] *J.Clin.Psychiatry* 60 Suppl 10:5-14: 5 - 14. (1999)

5 Shuck, M. E., Bock, J. H., Benjamin, C. W., Tsai, T. D., Lee, K. S., Slightom, J. L., and Bienkowski, M. J. Cloning and characterization of multiple forms of the human kidney ROM-K potassium channel *J.Biol.Chem.* 269: 24261 - 24270. (1994)

Taglialatela, M., Castaldo, P., Pannaccione, A., Giorgio, G., Genovese, A., Marone, G., and Annunziato, L. Cardiac ion channels and antihistamines: possible mechanisms of
10 cardiotoxicity *Clin.Exp.Allergy* 29 Suppl 3:182-9: 182 - 189. (1999)

Table 1: SEQ ID. NO. 1

Nucleotide sequence of TRK1

ATGCATTTTAGAAGAACGATGAGTAGAGTGCCACATTGGCATCTCTTGAAATACGATATAAAAAATCTTTCGGCC
 ATAAATTTTCGTGATTTTATTGCTCTATGTGGTCACTATTTTGCTCCAGTTAAAAAATATATCTTCCCCAGTTTAT
 5 CGCGGTTCACTACTTCTACACGATATCCCTGACATTAATAACTTCAATCCTGCTATATCCCATTAAAGAAACCAGA
 TACATTGATACATTGTTTTTAGCAGCGGGCGCAGTTACACAAGGTGGCTTAAATACTGTGGATATCAACAATCTAA
 GCTTATACCAACAATTTGTTCTGTATATCGTATGCTGCATATCAACACCAATTGCAGTTTCATAGTTGCTTGGCATT
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 GGCAACAAATTAAGAGAAGGCTTTCTACTGGTTCAATTGAGAAAAACAGCAGTAACAATGTTTCAGATAGAAAACC
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 40 CATTATCGAAAGGCTATAGAGTCCTTGTGCGCCTGTTTCAATCTGTTAGCACAGAAGTCTGAGATTGAGCGTTGT
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 45 ATATCTACAAAATCCTTTATCGGTGCCATTTAAGGAAACAGCTTTCATTTGACTTGTGGTTTCTATTTTAGGGT
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 TTTTGAAATTGTTAGCGCTTACGGTACAGTTGGGCTATCGCTAGGTTATCCGGACACCAACCAATCGTTTCAAGA
 CAGTTTACTACATTATCTAAGTTGGTGATCATAGCTATGCTGATCAGAGGCAAGAATAGAGGTCTACCATACTCAC
 TGGATCGTGCAATTATCTTGCTTAGTATAGACTTGAACATATTGACCACCTTGAGGGCATGAAATTGAAGAGACA
 50 GGCTAGAACCAATACAGAAGACCCAATGACGGAACATTTCAAGAGAAGTTTCACTGATGTGAAACATCGTTGGGGA
 GCTCTTAAGCGTAAGACCACACATTCCCGAAATCCTAAAAGGAGCAGCACAACGCTCTAA

Table 2: SEQ ID. NO. 2

Nucleotide sequence of TRK2

ATGCCAACAGCTAAGAGGACGTCATCCAGGGCTTCGTTGGCACTGCCCTTCCAGTTACGGTTGGTGCACAAGAAAT
 CATGGGGCCATCGGCTAAGAGACTTCATTTCCGGGTTCTTAAAAATCATGCAGACCCATTGCTAAATACGTTTCCC
 5 CAACTTCATCGTGGTGCACATATCTACCTGATCACGCTGTCGATTATCGGGTCCATTCTGTTATATCCGTGCAAG
 AACACGGCGTTCATCGATGTGCTATTTCTGGCTGCTGGAGCGTCTACACAGGGCGGGCTGGCCACCAAGAGCACTA
 ACGATTTCAACCTGTACCAGCAGATAGTGGTGTACGTCATTACATTGCTGTCCACGCCTATACTTATTCATGGGTT
 TTTGGCCTTTGTTCAGGCTGTATTGGTTTGAAGGTACTTCGACAACATTAGGGATATCTCCAAACAGAATTTTAAA
 CTAAGAAGGACCATGACGTTGCAACAAAGGGAACATTCGGGCAGCAGTGGCAATGCCGCTCGAAGTAGGAGTTTCA
 10 AGGACAACCTGTTCCGTGGGAAGTTTGTTCAGAGAAGACCCACGACAATCCGCTTCAGATGTGCCGATGGACTC
 TCCTGACACGTCCGCATTGTCTCAATCTCACCGTTGAATGTTTCTCTCTAAGGAGGAATCCAGTGACACGCAA
 AGCTCGCCTCCAACTTCTCAAGTAAGCGCCAACCTCAGACGTTGACCCAAGAGACATTTACAAATCGATAATGA
 TGCTACAAAAACAACAAGAGAAGAGCAACGCAAACTCCACGGATTCTTTTTCGAGCGAGACCAATGGACCCGCTTT
 CATTGTGCAGGAACGTCATGAGAGAAGAGCCCCCACTGCTCACTGAAACGCCATTCTGTCTGCCATCTTCTCAG
 15 GAATTGAACAAGCTAGCCCAGACGAAAAGTTTCCAGAAATTGCTTGGCTTGCGGAGAGATGAAGGTGACCATGACT
 ACTTTGACGGTGCTCCTCACAAATATATGGTCACCAAGAAGAAAAAATATCTAGAACGCAATCATGTAACATCCC
 AACGTATACTGCTTCACCGAGTCTTAAACCTCAGGCCAAGTAGTTGAAAATCATAGAACTTGGCCAAGTCGGCG
 CCTTCATCTTTTGTGATGAGGAGATGAGCTTTTCACCGCAAGAGTCTTTGAATTTACAGTTCCAAGCGCACCCGC
 CCAAACCAAAACGACGTGAAGGTGATATAGGCCACCCCTTCACCAGAACAATGAGCACCACACTATCTATCGTGGCA
 20 GCCAACCTTTGGCAGAACTCCGTCTTCATTGGACTCACAAGCAACAAAAGGAGGAACCTGGCGGTGTGCAATAT
 CGTGCTTTGAGATTGCTGTGCTGCATTCTCATGGTATACTACATCGGATTCAACATTTTGGCGTTTGTGACCATCG
 TTCCATGGGCCTGTACGAGGCACCACTACTCAGAGATTATTAGACGAAATGGAGTTTCTCCAACCTGGTGGGGGT
 TTTCACTGCAATGAGTGCATTACAGCAACTTGGGTCTGTCTTTGACCGCTGATTCAATGGTTTCTTTGATACTGCG
 CCGTATCCGCTGATTTTCATGATGTTCTTCATCATCATAGGCAATACAGGCTTCCCAATTATGTTACGATTTATCA
 25 TTTGGATCATGTTCAAGACCTCGAGAGACCTATCTCAGTTTAAAGGAAAGTCTTGGGTTTCTCTTGGATCATCCGCG
 CAGGTGTTTTACGTTGCTGTTTCCCGAGCGGCCACACATGGTGGCTGTTTACAACCTTAGTCGTCTTAAACGCTACG
 GATTGGATTCTTTTCATAAATCTTGGATTTCAACTCCGCTGTAGTAAGGCAGGTTGCTAAAGGTTATCGAGCTCTCA
 TGGGCCTCTTCCAGTCTGTATGCACAAGAAGTCTGGATTCAACGTTGTTGACTTAAGTAAATTACACCCGTCCAT
 TCAGGTGTCTTATATGCTAATGATGTACGTTTTCGGTCTGCGCTGGCGATTTCATTAGAAGAACGAATGTTTAT
 30 GAGGAGCAATCGTTGGGACTATACGATAGTGGACAAGATGACGAAAATATCACCCACGAAGACGATATAAAGGAAA
 CAGACCATGATGGCGAATCCGAAGAGCGAGACACTGTATCTACAAAGTCCAAGCCGAAGAACAGTCCCCAAAATC
 GTTTGTTGGTGCTCATTTGAGGAGGCAACTCTCTTTGATTTATGGTACCTATTCTCTGGATTATTTATAATATGC
 ATATGCGAGGGCAGAAAAATCGAAGACGTTAATAAACCTGATTTCAATGTCTTTGCTATATTGTTTGAAGTTGTTA
 GCGCTTATGGTACAGTGGGTTTGTCTATTGGGTTACCCAAACACCAACACATCACTATCTGCCAGTTTACCGTATT
 35 ATCGAAGCTAGTCATAATTGCCATGCTAATAAGAGGAAGAAATAGAGGTTTACCATACACTTTGGATCGTGCCATC
 ATGCTGCCAAGTGACAAACTGGAACAAATTGATCGTTTACAAGATATGAAAGCTAAGGGTAAGTTGTTAGCCAAAG
 TTGGTGAGGATCCAATGACTACTTACGTCAAAAAGAGATCCCAACAACTGAAAAAATAGCAACAAAGTTTGGGG
 GAAGCATTA

40 Table 3: SEQ ID NO. 3

Nucleotide sequence of TOK1

ATGACAAGGTTTCATGAACAGCTTTGCCAAACAAACGCTGGGATATGGCAATATGGCGACAGTGGAGCAAGAGAGCT
 CAGCTCAGGCTGTTGATTCTCATTCAAACAACACACCGAAGCAAGCTAAGGGTGTTCTTGACAGAGGAAGTAAAGGA
 TGCATTGCGGTTCCGGGACGAAAGAGTTAGTATTATTAATGCAGAGCCTTCTTCAACACTGTTCTGTTTGGTTT
 45 GTGGTTTCATGCTATTTCCCTGTGATTACTGCCTGCTTGGGTCCCGTAGCTAACACTATCTCGATAGCCTGTGTAG
 TTGAAAAATGGAGATCCTTAAAGAACAACCTCCGTGGTGACAAATCCACGAAGCAATGACACCGATGTTTTGATGAA
 TCAAGTAAAGACAGTTTTTGTATCCTCCTGGTATTTTGGCGTTAATATCATCTCTTTGGTACTGGGTTTTACGTCA
 AATATTATACTAATGCTACATTTTCAAGTAAGAAGTTGACGTATCTTAAATCTCAGTTAATAAATATAACAGGATGGA
 CAATAGCTGGAGGGATGCTTTTGGTGGACGTGATTGTATGCTCCTTGAATGACATGCCAGCATCTACAGTAAGAC
 50 TATCGGATTTTGGTTTGCCTGTATCAGTTCTGGTCTATATTTGGTATGCACCATTATTTTAAACAATACATTTTATT
 GGATATAAATTAGGAAAAATATCCTCCAACGTTCAACCTTTTGCCCAATGAAAGAAGTATCATGGCATACTACTGTAC
 TATTGTCTTTATGGTTGATTTGGGGTGCGGGTATGTTTAGCGGTTTATTGCACATCACTTACGGAAATGCATTATA
 TTTCTGCACGGTATCATTATTAACCGTGGGACTAGGTGACATCCTGCCCAAGTCGGTTGGCGCCAAAATCATGGTT

TTAATCTTTTCGCTATCTGGTGTGCTTGTGATGGGTTTAAATAGTGTATGACAAGATCCATCATTCAAAAAGTCCT
 CTGGCCCAATTTTCTTTTCCACAGAGTTGAAAAAGGCAGGTCCAAATCGTGGAACATTATATGGATAGTAGTAA
 AAATTTATCTGAAAGGGAAGCGTTCGACTTAATGAAGTGTATCCGACAAACGGCCTCAAGGAAGCAGCATTTGGTTT
 TCTTTGTGCGGTGACTATTGCAATTTTCATGGCTTTTGGTTATTGGGAGCTCTTGATTCAAATTCGCAGAAAAAT
 5 GGTCTGACTTCAATTGTATTTACTTTTGTCTTGTGCTTATTAACCATTTGGATACGGAGACTATGCTCCAAGGAC
 TGGTGACAGCCGTGCTTTTTTTGTGATTTGGGCGTTGGGAGCCGTGCCATTAATGGGGGCTATCCTATCTACAGTC
 GGTGATCTGTTGTTTGACATTTCCACTTCTCTGGATATTAAGATCGGTGAATCATTCAATAATAAAGTCAAGTCCA
 TCGTTTTTAATGGGCGTCAAAGAGCACTTCTCTTATGGTGAACACTGGAGAAATTTTCGAAGAATCTGACACAGC
 TGATGGTGTATCTGGAAGAAAATACAACGAGCTCACAATCCAGTCAAATTTCTGAATTC AACGATAATAATTCAGAA
 10 GAGAATGATTCTGGAGTGACATCCCCTCCTGCAAGCCTGCAAGAATCATTCTTTCATTATCAAAAGCATCTAGCC
 CAGAGGGAATACTTCTCTAGAAATATGTTTCTTCTGCTGAATATGCACTACAGGACTCGGGGACCTGTAATTTAAG
 GAACTTGCAAGAGCTACTTAAAGCCGTCAAAAACTACATCGGATATGCTGGCGGATAAAGATTACACACTTAGT
 TTTTCCGACTGGTCGTACATTATAAACTACATTTGAGGAACATTACAGATATTGAGGAGTACACACGCGGACCCG
 AATTTTGATATCACCTGATACGCCCCCTCAAGTTCCCGTTAAATGAACCTCATTCTTTTATGATGCTTTTCAA
 15 GAACATAGAAGAATTAGTTGGTAATCTAGTAGAAGACGAAGAGCTTTATAAAGTTATAAGCAAAAGAAAATTTTG
 GGTGAGCATAGAAAGACACTTTGA

Table 4: SEQ ID NO. 4

Nucleotide sequence of HERG1

20 ATGCCGGTGCGGAGGGGCCACGTGCGCGCGCAGAACACCTTCCTGGACACCATCATCCGCAAGTTTGAGGGCCAGA
 GCCGTAAGTTTCATCATCGCCAACGCTCGGGTGGAGAAGTGCAGCGCTCATCTACTGCAACGACGGCTTCTGCGAGCT
 GTGCGGCTACTCGCGGGCCGAGGTGATGCAGCGACCCCTGCACCTGCGACTTCTGCGACGGGCGCGCACGCAGCGC
 CGCGCTGCCGCGCAGATCGCGCAGGCACTGCTGGGCGCCGAGGAGCGCAAAGTGGAAATCGCCTTCTACCGGAAAG
 ATGGGAGCTGCTTCTATGTCTGGTGGATGTGGTGCCCGTGAAGAACGAGGATGGGGCTGTCTCATGTTCATCCT
 25 CAATTCGAGGTGGTGTATGGAGAAGGACATGGTGGGGTCCCCGGCTCATGACACCAACCACGGGGCCCCCACC
 AGCTGGCTGGCCCCGCGGCCAAGACCTTCCGCTGAAGCTGCCCGCGCTGCTGGCGCTGACGGCCCCGGGAGT
 CGTCGGTGCGGTGCGGGCGCGGGCGCGGGCGCCCCGGGGGCCGTGGTGGTGGACGTGGACCTGACGCCCCGC
 GGCACCCAGCAGCGAGTGCCTGGCCCTGGACGAAGTGACAGCCATGGACAACCACGTGGCAGGGCTCGGGCCCCGC
 GAGGAGCGGCGTGCCTGGTGGGTCCCGCTCTCCGCCCCGACGCGCGCCCGGCCAGCTCCCATCGCCCCGGGCGC
 30 ACAGCCTCAACCCCGACGCTCGGGCTCCAGCTGCAGCCTGGCCCGGACGCGCTCCCGAGAAAGCTGCGCCAGCGT
 GCGCCGCGCCTCGTCCGCGGACGACATCGAGGCCATGCGCGCCGGGGTGCTGCCCGCGCCACCGCGCCACGCCAGC
 ACCGGGGCCATGCACCCACTGCGCAGCGGCTTGCTCAACTCCACCTCGGACTCCGACCTCGTGCGCTACCGCACCA
 TTAGCAAGATTCCCCAAATCACCTCAACTTTGTGGACCTCAAGGGCGACCCCTTCTTGGCTTCGCCCACCAAGTGA
 CCGTGAGATCATAGCACCTAAGATAAAGGAGCGAACCCACAATGTCACTGAGAAGGTCAACCAAGTCTGTCCCTG
 35 GCGCGCGCTGCTGCCTGAGTACAGCTGAGGACCCGCGCATCCACCGCTGGACCATCTGCAATTACGCCCTT
 TCAAGGCCGTGTGGGACTGGCTCATCTGCTGCTGGTCACTACACGGCTGTCTTCAACCCCTACTCGGCTGCCTT
 CCTGCTGAAGGAGACGGAAGAAGGCCCGCTGCTACCGAGTGTGGCTACGCCGCGAGCGCTGGCTGTGGTGGAC
 CTCATCGTGACATCATGTTTATTGTGGACATCTCATCAACTCCGCACCACCTACGTCAATGCCAACGAGGAGG
 TGGTCAGCCACCCCGGCCGATCGCCGTCCACTACTTCAAGGGCTGGTTCTCATCGACATGGTGGCCGCCATCCC
 40 CTTTCGACCTGCTCATCTTCGGCTCTGGCTCTGAGGAGCTGATCGGGCTGCTGAAGACTGCGCGGCTGCTGCGGCTG
 GTGCGCGTGGCGCGGAAGCTGGATCGCTACTCAGAGTACGGCGCGGCCGTGCTGTTCTTGCTCATGTGCACCTTTG
 CGCTCATCGCGCACTGGCTAGCCTGCATCTGGTACGCCATCGGCAACATGGAGCAGCCACACATGGACTCACGCAT
 CGCTGGCTGTCACAACCTGGGCGACCATAGGCAAAACCTACAACAGCAGCGGCTGGGCGGCCCCCTCCATCAAG
 GACAAGTATGTGACGGCGCTCTACTTCACTTACAGCAGCTTCAACAGTGTGGGCTTGGGCAACGCTCTCTCCACA
 45 CCAACTCAGAGAAGATCTTCTCCATCTGCGTCATGCTCATTTGGCTCCCTCATGTATGCTAGCATCTTCGGCAACGT
 GTCGGCCATCATCCAGCGGCTGTACTCGGGCACAGCCCGCTACACACACAGATGCTGCGGGTGGGGAGTTTCATC
 CGCTTCCACAGATCCCCAATCCCCTGCGCCAGCGCCTCGAGGAGTACTTCCAGCACGCCTGGTCTTACACCAACG
 GCATCGACATGAACGCGGTGCTGAAGGGCTTCCCTGAGTGCCTGCAGGCTGACATCTGCCTGCACCTGAACCGCTC
 ACTGCTGCAGCACTGCAAACCTTCCGAGGGGCCACCAAGGGCTGCCTTCGGGCGCTGGCCATGAAGTTCAAGACC
 50 ACACATGCACCCGCCAGGGGACACACTGGTGCATGCTGGGGACCTGCTCACCAGCCCTGTACTTCATCTCCCGGGGCT
 CCATCGAGATCCTGCGGGGCGACGTGCTGCTGGCCATCCTGGGGAAGAATGACATCTTTGGGGAGCCTCTGAACCT
 GTATGCAAGGCCTGGCAAGTCGAACGGGGATGTGCGGGCCCTCACCTACTGTGACCTACACAAGATCCATTCGGGAC
 GACCTGCTGGAGGTGCTGGACATGTACCTTCTCCGACCACTTCTGGTCCAGCTGGAGATCACCTTCAACC
 TGCGAGATACCAACATGATCCCAGGCTCCCCCGGCAGTACGGAGTTAGAGGGTGGCTTCACTGCGGCAACGCAAGCG
 55 CAAGTTGTCTTCCGCGAGGCGCACGGACAAGGACACGGAGCAGCCAGGGAGGTGTCGGCCTTGGGGCCGGGCGG

GCGGGGGCAGGGCCGAGTAGCCGGGGCCGGCCGGGGGGGCGGTGGGGGGAGAGCCCGTCCAGTGGCCCCCTCCAGCC
 CTGAGAGCAGTGAGGATGAGGGCCAGGCCGAGCTCCAGCCCCCTCCGCCTGGTGCCCTTCTCCAGCCCCAGGCC
 CCCCAGAGAGCCGCCGGGTGGGGAGCCCCTGATGGAGGACTGCGAGAAGAGCAGCGACACTTGCAACCCCTGTCA
 5 GGCGCCTTCTCAGGAGTGTCCAACATTTTCAGCTTCTGGGGGGACAGTCGGGGCCGCGCAGTACCAGGAGCTCCCTC
 GATGCCCCCGCCCCACCCCCAGCCTCCTCAACATCCCCCTCTCCAGCCCGGGTCGGCGGGCCCCGGGGCGACGTGGA
 GAGCAGGCTGGATGCCCCTCAGCGCCAGCTCAACAGGCTGGAGACCCGGCTGAGTGCAGACATGGCCACTGTCTTG
 CAGCTGCTACAGAGGCAGATGACGCTGGTCCC GCCCCTACAGTGCTGTGACCACCCGGGGCCTGGCCCCACTT
 CCACATCCCCGCTGTTGCCCGTCAGCCCCCTCCCCACCCTCACCTTGGACTCGCTTTCTCAGGTTTCCAGTTCAT
 10 GCGGTGTGAGGAGCTGCCCCGGGGGCCCCAGAGCTTCCCAAGAAGGCCCCACACGACGCCTCTCCCTACCGGGC
 CAGCTGGGGGCCCTCACCTCCCAGCCCCTGCACAGACACGGCTCGGACCCGGGCAGTTA

Table 5: SEQ ID NO. 5

15 Nucleotide sequence of K_y1-5

ATGGAGATCGCCCTGGTGCCCCCTGGAGAACGGCGGTGCCATGACCGTCAGAGGAGGCGATGAGGCCCGGGCAGGCT
 GCGGCCAGGCCACAGGGGGAGAGCTCCAGTGTCCCCCGACGGCTGGGCTCAGCGATGGGCCCAAGGAGCCGGCGCC
 AAAGGGGGCGCGCGCAGAGAGACCGGACTCGGGAGTGCGGGCCCTTGCTTCCGCTGCCGGACCCGGGAGTGCGGGCC
 20 TTGCCTCCGCTGCCAGAGGAGCTGCCACGGCCTCGACGGCCGCTCCCGAGGACGAGGAGGAAGAAGGCGATCCCCG
 GCCTGGGCACGGTGGAGGACCAGGCTCTGGGCACGGCGTCCCTGCACCACCAGCGCGTCCACATCAACATCTCCGG
 GCTGCGCTTTGAGACGCAGCTGGGCACCCTGGCGCAGTTCCCCAACACACTCCTGGGGGACCCCGCCAAGCGCCTG
 CCGTACTTCGACCCCCCTGAGGAACGAGTACTTCTTCGACCGCAACCGGCCAGCTTCGACGGTATCCTCTACTACT
 ACCAGTCCGGGGGGCCGCTGCGAGGGGTCAACGTCTCCCTGGACGTGTTGCGGGACGAGATACGCTTCTACCAGCT
 25 GGGGGACGAGGCCATGGAGCGCTTCCGCGAGGATGAGGGCTTCATTAAAGAAGAGGAGAAGCCCTGCCCCGCAAC
 GAGTTCCAGCGCCAGGTGTGGCTTATCTTCAGTATCCGGAGAGCTCTGGGTCCGCGGGGCCATCGCCATCGTCT
 CGGTCTTGGTTATCCTCATCTCCATCATCACCTTCTGCTTGGAGACCCTGCCTGAGTTCAGGGATGAACGTGAGCT
 GCTCCGCCACCTTCCGGCGCCCCACCAGCCTCCCCGCGCCCCCGCCCTGGGGCCAACGGCAGCGGGGTGATGGCCCCC
 GCCTCTGGCCCTACGGTGGCACCGCTCCTGCCCAGGACCCTGGCCGACCCCTTCTTCATCGTGGAGACCACGTGCG
 30 TGATCTGGTTACCTTCGAGCTGCTCGTGCGCTTCTTCGCCTGCCCCAGCAAGGCAGGGTCTCCCGGAACATCAT
 GAACATCATCGATGTGGTGGCCATCTTCCCCTACTTCATCACCTGGGCACCGAAGTGGCAGAGCAGCAGCCAGGG
 GCGGAGGAGGCGGCCAGAATGGGCAGCAGGCCATGTCCCTGGCCATCCTCCGAGTCATCCGCTGGTCCGGGTGT
 TCCGCATCTTCAAGCTCTCCCGCCACTCCAAGGGGCTGCAGATCCTGGGCAAGACCTTGACGGCCTCCATGAGGGA
 GCTGGGGCTGCTCATCTTCTTCTTCTTCATCGGGGTGATCCTCTTCTCCAGTGCCGTCTACTTCGAGAGGCTGAC
 35 AACCAGGGAACCCATTTCTCTAGCATCCCTGACGCCTTCTGGTGGGCAGTGGTCACCATGACCACTGTGGGCTACG
 GGGACATGAGGCCCATCACTGTTGGGGGCAAGATCGTGGGCTCGCTGTGTGCCATCGCCGGGGTCTCACCATTGC
 CCTGCCTGTGCCCCGTCATCGTCTCCAACCTTCAACTACTTCTACCACCGGAAACGGATCACGAGGAGCCGGCAGTC
 CTTAAGGAAGAGCAGGGCACTCAGAGCCAGGGGCCGGGGCTGGACAGAGGAGTCCAGCGGAAGGTGACGGGGAGCA
 GGGGATCCTTCTGCAAGGCTGGGGGACCCTGGAGAATGCAGACAGTGCCCGAAGGGGCAGCTGCCCCCTAGAGAA
 40 GTGTAACGTCAAGGCCAAGAGCAACGTGGACTTGCGGAGGTCCCTTTATGCCCTCTGCCTGGACACCAGCCGGGAA
 ACAGATTTGTGA

Table 6: SEQ ID NO. 6:

45

Nucleotide sequence of IRK1

ATGGGCAGTGTGCGAACCAACCGCTATAGCATTGTCTCTTCGGAAGAGGACGGCATGAAGTTGGCCACCATGGCAG
 TTGCCAATGGCTTTGGGAATGGGAAAAGTAAAGTCCACACTCGGCAACAGTGTAGGAGCCGCTTTGTGAAGAAAGA
 50 TGGCCACTGTAATGTTTCAGTTCATCAACGTTGGGGAAAAGGGACAACGGTACCTTGCTGACATTTTTACTACGTGT
 GTGGACATTGCTGGCGGTGGATGCTGGTTATCTTTGCTTAGCTTTTGTCTCTCGTGGCTGTTTTTGGCTGTG
 TGTTTTGGCTGATAGCTTTGCTCCATGGAGATCTGGATGCATCTAAGGAGAGCAAAGCCTGTGTGTCTGAGGTCAA
 CAGCTTCACAGCTGCCTTTCTTTCTCCATTGAGACCCAGACAACCATCGGCTATGGGTTCGATGTGTACGGAT
 GAATGCCCCGATTGCGGTGTTTCATGGTTGTGTTCCAGTCAATTGTGGGCTGCATTATTGATGCTTTTATCATTGGTG
 55 CCGTCATGGCAAAGATGGCAAAGCCAAAGAAAGAAATGAGACTCTGTCTTCAGTCACAATGCTGTGATTGCCAT
 GAGAGATGGCAAAGCTGTGTTTGTGATGTGGCGAGTAGGCAACCTTCGGAAAAGCCACTTGGTAGAAGCTCATGTTTCA
 GCCCAGCTCCTCAAATCCAGAATTACTTCTGAAGGGGAATACATCCCCCTGGATCAAATAGACATCAATGTTGGCT

TTGACAGTGGAATTGACCGTATATTTCTGGTATCCCCAATCACTATTGTCCATGAAATAGATGAAGATAGTCCTTT
 ATATGATTTGAGCAAGCAGGACATTGATAATGCAGACTTTGAAATTGTTGTGATACTAGAAGGCATGGTGGGAAGCC
 ACTGCCATGACAACACAGTGTCTAGTTCTTATTTGGCCAACGAGATCCTTTGGGGCCACCGCTATGAGCCAGTGC
 TCTTTGAGGAGAAGCACTACTATAAAGTGGACTATTTCGAGGTTTCATAAGACTTACGAAGTACCCAACACTCCCCT
 5 TTGTAGTGCCAGAGACTTAGCAGAAAAGAAATATATTCTCTCAAATGCTAACTCATTTTGCTATGAAAATGAAGTT
 GCCCTTACAAGCAAAGAGGAAGATGACAGTGAAAATGGGGTTCCAGAAAGCACCAGTACAGACACACCTCCTGACA
 TCGACCTTCACAACCAGGCAAGTGACCTCTAGAGCCCAGACCCTTACGGCGAGAATCGGAGATATGA

10 TABLE 7

SEQ ID NO. 31 human ROMK2 (Genbank accession number U12542)

15 ATGTTCAAACATCTTCGGAAATGGGTCGTCCTCTTTTGGGCATTCTCGGCAAAGAGCAAGGCTAGTCTCCA
 AAGATGGAAGGTGCAACATAGAATTTGGCAATGTGGAGGCACAGTCAAGGTTTATATTCTTTGTGGACATCTGGAC
 AACGGTACTTGACCTCAAGTGGAGATACAAAATGACCATTTTCATCACAGCCTTCTTGGGGAGTTGGTTTTTCTTT
 GGTCTCCTGTGGTATGCAGTAGCGTACATTACAAAGACCTCCCGGAATTCCATCCTTCTGCCAATCACACTCCCT
 GTGTGGAGAATATTAATGGCTTGACCTCAGCTTTTCTGTTTTCTCTGGAGACTCAAGTGACCATTGGATATGGATT
 20 CAGGTGTGTGACAGAACAGTGTGCCACTGCCATTTTTCTGCTTATCTTTCAGTCTATACTTGGAGTTATAATCAAT
 TCTTTTCATGTGTGGGGCCATCTTAGCCAAGATCTCCAGGCCCAAAAAACGTGCCAAGACCATTACGTTTCAGCAAGA
 ACGCAGTGATCAGCAAACGGGGAGGGAAGCTTTGCCTCCTAATCCGAGTGGCTAATCTCAGGAAGAGCCTTCTTAT
 TGGCAGTCACATTTATGGAAAGCTTCTGAAGACCACAGTCACTCCTGAAGGAGAGACCATTATTTTGGACCAGATC
 AATATCAACTTTGTAGTTGACGCTGGGAATGAAAATTTATTCTTCATCTCCCCATTGACAATTTACCATGTCATTG
 25 ATCACAACAGCCCTTTCTTCCACATGGCAGCGGAGACCCTTCTCCAGCAGGACTTTGAATTAGTGGTGTGTTTTAGA
 TGGCACAGTGGAGTCCACCAGTGCTACCTGCCAAGTCCGGACATCCTATGTCCAGAGGAGGTGCTTTGGGGCTAC
 CGTTTTGCTCCCATAGTATCCAAGACAAAGGAAGGAAATACCGAGTGGATTTCCATAACTTTAGCAAGACAGTGG
 AAGTGGAGACCCCTCACTGTGCCATGTGCCTTTATAATGAGAAAAGATGTTAGAGCCAGGATGAAGAGAGGCTATGA
 CAACCCCAACTTCATCTTGTGAGAAGTCAATGAAACAGATGACACCAAAATGTAA

30

HERG in $\Delta trk1\Delta trk2\Delta tok1$ in DPM -HIS medium with 0.5 mM CsCl as activator after 38 hours growth. Starting culture 0.03 OD. Detection OD620nm.

	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Mean growth	St. Dev.
Inhibitors (30 μM)						
Terfenadine	0.006	0.006	0.005	0.007	0.006	0.0
Pimozide	0.004	0.004	0.005	0.005	0.0045	0.0
Diphenhydramine	0.095	0.151	0.17	0.186	0.1505	0.04
Ziprasidone	0.006	0.01	0.012	0.015	0.01075	0.0
Fexofenadine	0.082	0.144	0.159	0.156	0.13525	0.0
Sertindole	0.007	0.004	0.007	0.005	0.00575	0.0
Loratadine	0.024	0.016	0.062	0.014	0.029	0.0
DMSO control	0.162	0.163	0.136	0.146	0.15175	0.0

Wild-type cells in DPM medium with 5 mM or 80 mM KCl after 24 hours growth. Starting culture 0.01 OD. Detection at OD 620 nm.

[illegible]

TABLE 9

LacZ in wild-type cells in DPM -HIS/-TRP medium with 0.5 mM CsCl as activator after 24 hours growth; detection with the TROPIX kit. ASSAY 1						
Inhibitors (30 μ M)	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Mean growth	St. Dev.
Terfenadine	4497	4481	5058	5381	4854.25	441.936176
Pimozide	357.4	747.9	804.6	585.4	623.825	200.443648
Diphenhydramine	2806	3181	4178	4864	3757.25	937.881789
Ziprasidone	55.32	70.29	70.3	77.18	68.2725	9.22481933
Fexofenadine	3326	2938	3377	4659	3575	748.783458
Serindole	4165	2099	4588	3069	3480.25	1121.45304
Loratadine	4905	5141	1857	3266	3792.25	1536.17173
DMSO control	3172	4129	4984	5077	4340.5	888.190858
LacZ in wild-type cells in DPM -HIS/-TRP medium with 0.5 mM CsCl as activator after 24 hours growth; detection with TROPIX-kit. ASSAY 2						
Inhibitors (30 μ M)	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Mean growth	St. Dev.
Terfenadine (0.5 Cs)	3439	3795	3698	3388	3580	197.394698
Pimozide (0.5 Cs)	905	2176	496.5	573.4	1037.725	779.2749
Diphenhydramine (0.5 Cs)	3468	2980	3062	3561	3267.75	289.383684
Ziprasidone (0.5 Cs)	62.52	44.3	49.71	51.87	52.1	7.64158361
Fexofenadine (0.5 Cs)	3533	3502	3661	3569	3566.25	68.8446318
Serindole (0.5 Cs)	3992	3076	3972	2782	3455.5	619.738386
Loratadine (0.5 Cs)	3553	1965	3590	2478	2896.5	807.211042
DMSO control (0.5 Cs)	3520	3218	3460	3087	3321.25	203.540946

		80 mM KCl	Std
	3,959	3,76275	0,30362738
	3,441	3,51975	0,10563262
	0,606	0,71	0,09588535
	3,947	3,555	0,36347856

TABLE 12

p423GPD (YM194) and p423GPD-ROMK2 (YM256) in $\Delta trk1\Delta trk2\Delta tok1$ in DPM -HIS 5 mM KCl, pH 6.5 after 24 hours growth; starting OD 0.01. Averages				
	194 SD		256 SD	
DMSO (0.5%)	0.023	0.0036	0.19	0.013
Cells	0.028	0.0012	0.23	0.011
2 mM RbCl	0.048	0.0052	0.44	0.033
Signal to noise ratio				
S/N				
DMSO (0.5%)	8.2			
Cells	8.3			
2 mM RbCl	8.46			

Table 13: SEQ ID NO. 31 – Nucleotide sequence of p423 GPD-hROMK2
(Accession No. U 12542)

gacgaaagggcctcgtgatacgccctatTTTTataggttaatgtcatgataataatggtttcttagatgatccaatatcaaagg
 5 aaatgatagcattgaaggatgagactaatccaattgaggagtggcagcatatagaacagctaaagggtagtgctgaag
 gaagcatacgataccccgcatggaatgggataatcacaggaggtagactacctttcatcctacataaataagacg
 catataagtacgcatttaagcataaacacgcactatgccgttcttctcatgtatatatatatacaggcaacacgcagatata
 ggtgcgacgtgaacagtgaagctgtatgtgcgcagctcgcgttgcatTTTcggaagcgcctgTTTcggaacgcTTTgaagt
 tcctattccgaagttcctattctctagaaagtataggaacttcagagcgcTTTgaaaacaaaagcgcctctgaagacgca
 10 cttcaaaaaacaaaaacgcaccggactgtaacgagctactaaaaatattgcaataccgcttcacaaaacattgctca
 aaagtatctctttgctatatatctctgtgctatatccctatataacctacccatccacctttcgctcctgaactgcatctaaact
 cgacctctacattTTTTatgtttatctctagtattactctttagacaaaaaaattgtagtaagaactattcatagagtgaatcgaa
 aacaatacgaaaatgtaaacatttcctatacgtagtatatagagacaaaaatagaagaaaccgttcataattttctgaccaa
 tgaagaatcatcaacgcctatcactttctgttcacaaagtatgcgcaatccacatcggatatagaataaatcggggatgccttt
 15 atcttgaaaaaatgcacccgcagcttcgctagtaatcagtaaaccgcgggaagtggagtcaggcTTTTtatggaagaga
 aaatagacaccaaagtagccttcttaaccttaacggacctacagtgcaaaaagttatcaagagactgcattatagagc
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